SOME ASPECTS OF AMMONIA TOXICITY ON THE GILL PATHOLOGY OF CARP (*Cyprinus carpio* L.) AND TROUT (*Salmo gairdneri* R.)

by

LAKSHMIKANTHAM P. KOTHANUR
BFSc.,MSc.,(Mariculture)

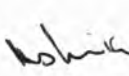
Thesis submitted to the University of Stirling
for the degree of Doctor of Philosophy

Institute of Aquaculture
University of Stirling
Stirling, Scotland
United Kingdom.
JULY 1989

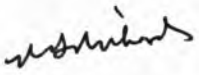
To my beloved
Father & Mother

DECLARATION

This thesis embodies the results of scientific experimental investigations carried out by Mr.K.P.Lakshmikantham at the Institute of Aquaculture University of Stirling, during the period 1985 - 1988. The thesis has been composed independently by Mr K.P.Lakshmikantham and no part of this work has been submitted for any other degree.

Candidate: 

(K.P.Lakshmikantham)

Principal Supervisor: 

(Dr. R.H.Richards)

Institute of Aquaculture
University of Stirling
Stirling

Acknowledgements

My sincere thanks are due to my supervisor Dr. R.H.Richards for his guidance, support and constructive criticism throughout the period of this study. I thank Professor R.J.Roberts, Director of the Institute of Aquaculture, for his encouragement.

I am grateful to Dr.A.D.Pickering of the Fresh Water Biological Association, Ambleside. Windermere, and Dr.Brian Eddy of the department of the Biological Sciences, of the University of Dundee, for their extensive discussion and advice.

In the course of this study I have been greatly benefited by the technical expertise of various technical staff and my sincere thanks in particular are due to Mr. Paul Thompson of the histology division, Mr. William Struthers of the water quality division, Mr.Stewart Miller of the microbiology division, of the Institute of Aquaculture, and Mr. Jim Purvis of the electron microscopical unit of the department of the Biological Sciences. I also thank Mr. Lewis Taylor for his generous help in photographic work.

Help and assistance of Laura Cummings, Peter Southgate, Richard Collins, Gary Nash, Krishen Rana, Betty Stenhouse, Charley Harrower, Ian MacGovan, Keith Ransom, and Jean Brown is greatly acknowledged with sincere gratitude to all other staff members of the Institute of Aquaculture.

Much of the text that has gone in to this thesis has been typed by Shadow Bostock and the same is acknowledged thankfully.

I thank all my fellow Ph.D students and friends for their support and help throughout this study period.

The friendship and fellowship of the members of the Stirling Junior Chamber has been most memorable and I sincerely thank all the members for their generosity.

Finally my thanks are to the Government of India for providing a fellowship enabling me to pursue this study at Stirling, and also to the Committee of the Principals and Vice Chancellors of the Universities of the United Kingdom for the award of a partial grant in the course of this study.

CONTENTS

ABSTRACT

The toxic effects of ammonia on the gill structure of common carp, Cyprinus carpio L., and rainbow trout Salmo gairdneri R., were investigated by both light microscopic histological studies, scanning and transmission electron microscopical studies. Experimental procedures adopted included the use of standard dilution water under constant temperature conditions and commonly used buffers to stabilize the pH, while additional experiments were also conducted in non buffered dilution water.

The common pathological changes noted for both carp and trout included severe chloride cell proliferation, excessive mucous secretions and an infiltration of large numbers of eosinophilic granular cells into the gill epithelia. Prolonged exposure to high concentrations of ammonia resulted in progressive necrosis of chloride, mucous,and eosinophilic granular cells. Apoptotic changes indicative of cell death in the gill epithelium included the appearance of condensed darkened cells,and cells with nuclear changes such as pyknosis and karyorrhexis. Dilation of mitochondria, loss of mitochondrial matrix, and appearance of dark inclusions were particularly noted in the carp gill chloride cells.Damage to the mucous cells consisted of premature occlusion of the mucous vesicles and flocculation of the mucous material within the mucous cells, resulting in progressive vacuolations, necrosis and thus a reduction in the stainable population of mucous cells.

However, specific lesion types varied between the carp and trout.

Carp gills developed massive hyperplastic and hypertrophic lesions comprising of grossly swollen gill epithelial and infiltration cells under buffered experimental conditions,while such lesions were not only less extensive in non buffered experimental conditions but the tissue changes were dominated by the loosening and sloughing of the gill epithelium. Discrepancies in the histological alterations were however evident as a consequence of the presence of buffers and hence the very validity of the recommendation and use of buffer chemicals in toxicity testing was questioned.

In the case of trout no hyperplastic lesions such as those seen in carp were not seen but an increased inter and intra cellular oedema was characteristic. Under normal water hardness conditions trout gills apparently showed no variations due to the presence of different buffers or in the non-buffered water conditions.But under high hardness conditions and in the presence of buffer, the gill, lesions were more extensive and necrotic.

The results suggest that while ammonia brings about necrotic changes in the gill tissues of both, carp and trout, the lesions are more necrotic in carp than in trout.These results also indicate that the indiscriminate use of buffers and variations in the water quality conditions might have been the cause of wide variations in the reported toxicity values of ammonia to fish in addition to the reported differences in pathology in the available published literature.

The recent reports of a small number of workers that ammonia *per se* does not cause any pathology is not supported by the present investigations.

CHAPTER 1

INTRODUCTION

In intensive aquaculture facilities and hatchery systems external irritants have been considered as the most frequent causes of significant gill pathological changes. The gills are among the most delicate structures of the teleost body, and their vulnerability and liability to damage due to external irritants is thus greater due to the fact that they are in compulsory contact with the surrounding medium (Roberts, 1978). The disease syndromes associated with the gills are often described as of complex aetiology and it is very often difficult to attribute a specific lesion to a particular irritant or a specific group of irritant chemicals as being responsible for a definite and characteristic reaction. According to Roberts (1978), "because of its relative simplicity of structure only a limited number of reactions can be manifested by the diseased gills". Moreover, effects that may be of chemical or irritant origin could very soon be obscured by stress provoked infections with facultative pathogens.

The accumulations of nitrogenous excretory products have long been considered as the major causes of functional or structural disorders in fish cultivation, and this has been particularly attributed to ammoniacal gill excretion, especially under over crowded conditions (Post, 1983). Thus the toxicity of ammonia on fish, and in particular its effects on gills, has been extensively studied. However, such studies have been more common in salmonids than in cyprinids or any other " coarse" fish.

From the available literature it is also clearly established that under given environmental conditions the amount of ammonia required to produce a lethal effect can vary to a great extent from a more sensitive fish to a more tolerant fish. Thus, for example, the amount of unionized ammonia expressed in terms of LC50 values for a more resistant species such as carp is several hundred percent higher than that required for a less tolerant fish such as rainbow trout to produce the same effect.However the mechanisms involved in such varied tolerance capabilities remain poorly understood.

While the earlier literature (Burrows, 1964) pointed out that ammonia at a concentration as low as 0.006 mgl^{-1} as unionised ammonia (UIA) was sufficient to cause severe gill hyperplasia in a salmonid fish, later works have disagreed with such a view (Bullock, 1972; Smart, 1976). Further recent studies (Mitchell and Cech, 1983; Daoust and Ferguson, 1984) involving the more resistant channel catfish and more susceptible rainbow trout at increased sub lethal levels of ammonia failed to relate any kind of lesions to ammonia. Based on the experimental results involving the exposure of rainbow trout to 0.4 mgl^{-1} UIA for 80 days Daoust and Ferguson (1984) declared that ammonia *per se* does not cause any gill changes. Subsequent studies (Thurston *et al.*,1984; Klontz *et al.*,1985) have further provided evidence for severe gill lesions in trout involving a variety of types of lesion.

Most of the studies describing normal gill structural features at both the light microscopical and ultrastructural levels, and much of the information on the pathology of the gill tissues attributable to ammonia in general, comes from experimental investigations on more sensitive salmonid fishes. There is little information available both on the normal gill structural features and on the toxic effects of ammonia on fish such as carp.

Much of the histopathology associated with ammonia toxicity is based on light microscopical investigations and the lesions most often described are hyperplasia, hypertrophy and telangiectasis. In spite of consistent and repetitive reporting of gill hyperplasia and hypertrophy the specific cell types involved in these two distinctively different reactions have not been specified or differentiated.Little advantage has been taken of the high resolution capabilities of either scanning or transmission electron microscopy for examining the affected branchial epithelium, or any particular branchial cells involved in the pathological processes.

In spite of the obvious differences in the tolerance capabilities of different fish species to ammonia, no comparative studies seem to have been undertaken to relate the structural features of these two groups of fish under identical or uniform water quality conditions, prior to and after the experimental ammonia exposure. Water quality parameters other than ammonia, pH and temperature are often not taken into account, and the use of a wide variety of dilution waters in

a wide variety of experiments involving different fish species, has made comparison of individual studies involving different fish species difficult.

Although many of the ammonia toxicity studies have used buffers extensively to regulate the pH, there is no uniformity in either the chemical substances used or the concentrations adopted in individual experiments, apart from variations in parameters such as hardness or alkalinity.

In the light of these aforementioned disparities, the present investigation was designed to verify the possible occurrence of any gill lesions, and if so, to provide a comparison as to how these lesions differed in two selected phylogenetically different fish species of utmost commercial importance with different ammonia tolerance capabilities.

It was also intended to examine the gills under the electron microscope wherever appropriate to elucidate in more detail the lesions when present. Since the water quality, pH and temperature are the important determining factors in ammonia toxicity studies, recommended standard dilution water and buffers that have been used by other workers were adopted to provide uniform and stable water quality conditions.

Common carp, Cyprinus carpio.L. and rainbow trout Salmo gairdneri.R. were used as experimental

CHAPTER 2

A REVIEW OF LITERATURE OF THE SOURCES OF AMMONIA IN NATURAL WATERS, ITS SYNTHESIS AND EXCRETION IN FISH, DYNAMICS IN AQUATIC AND BIOLOGICAL FLUIDS, METHODS OF MEASUREMENT AND ITS TOXICITY TO FISH

Ammonia is widely recognised as one of the most common pollutants present in aquatic environments (Lloyd and Orr,1969); USEPA, (1985) It is present in most natural waters as a biological degradation product of proteins. Decomposition of bottom sediments , organic constituents in the mud, decaying algae and aquatic vegetation following seasonal blooms are the other biological factors that increase the ammonia content of natural waters. However, the concentration of these sources may be small and subsequent conversion to nitrate through nitrification may take place (Alabaster and Lloyd, 1980) rendering their concentrations so low as to be of no significant danger in open natural waters.

High concentrations of ammonia are often associated with human sources and of these, the municipal or sewage effluents are the most common. Other important sources of ammonia pollution are industrial effluents such as from coke and fertilizer manufacturing, energy development processes such as oil shale retorting, coal gasification and coal liquification (Thurston and Russo, 1983). Other frequent sources of ammonia are agricultural inputs and effluents such as feed lots, fertilizers, silage and manures (Alabaster and Lloyd, 1980). Ammonia has been identified as one of the most common pollutants occurring in both British and European polluted inland waters along with copper, zinc, phenol,and cyanide (Brown, Shurben, and Shaw, 1970; Holden and Lloyd, 1972).

Ammonia and ammonium salts have also been used in fish pond fertilization, aquatic weed control (Alabaster and Lloyd, 1980), and in the eradication of undesirable fishes from culture ponds

(Subramanian, 1983), although the use of ammonia in the treatment of fish for ectoparasites has been abandoned largely due to its toxicity (Kabata, 1985).

In intensive fish farming, apart from the water source, the fish themselves are a major source of ammonia, as ammonia forms one of the chief end products of protein metabolism in all aquatic invertebrates and teleosts.Ammonia comprises as much as 60 to 90 percent of the total nitrogen excreted by fish (Smith,1929; Wood, 1958; Fromm, 1963). According to Vellas - Clos(1973),as much as 93.9% of the total nitrogen is excreted as ammonia in the carp, (Cyprinus carpio).

The amount of ammonia excreted by fish has been found to be proportional to the feeding rate and thus a doubling of the feed given to fish will roughly double the ammonia produced, as has been demonstrated by Tucker *et al.*(1979), in the case of channel catfish. Fischer (1977) has also shown the quantity of ammonia excreted by cyprinids to be directly proportional to the feeding rate. Very recently, Kaushik (1980) has demonstrated that an increase in the feeding rate from one meal to satiation to two meals to satiation per day resulted in almost a doubling in the rate of excretion of ammonia amounting to an increase from 325 mg NH_3-N/kg body weight/day to 581 mg NH_3-N/kg body weight/day respectively. It was shown that about 30-35 percent of the total nitrogen consumed was lost through ammonia as metabolic waste. Similar ammonia production rates were also noted in the case of rainbow trout, Salmo gairdneri, and the nitrogen loss as ammonia and urea accounted for between 48 and 52% of nitrogen consumed under different feeding conditions (Kaushik, 1980).

Under intensive fish rearing conditions a maximum total ammonia excretion rate of 700 mg NH_3-N/kg fish/day was reported for rainbow trout held at 10°C (Smart, 1981). Thus, it seems reasonable to expect considerably higher ammonia concentrations in intensive fish farming conditions fed at high rates, than those at moderate conditions of feeding. If accumulated, such metabolically produced ammonia might have severe adverse effects on fish.Additionally, depending on the feeding rates varying amounts of nitrogen may also reach the water as uneaten or undigested feed and in fish faeces. The nitrogen in these substances is probably mineralized fairly rapidly (Tucker, 1985) releasing ammonia into the water.Depending on the location of the fish farm, source of water, and the type of farming involved, some or all of these sources of ammonia may contribute to its toxicity and hence be important to the fish farming industry as well as for natural fisheries.

SOURCES AND SYNTHESIS OF AMMONIA IN FISH

The major sources of tissue ammonia are from transdeamination that may particularly occur in liver, kidney and muscle, and the AMP deaminase cycle occurring particularly in muscle. The relative importance probably depends on the physiological and nutritional state of the animal, with transdeamination becoming important when amino acids break down, and are used as the main source of energy for general metabolic processes, except in muscle where aspartate aminotransferase and AMP cycle may be the major route of amino acid utilization (Watts and Watts 1974).

As proteins within all organisms are in a constant state of turnover, either continually being synthesized or degraded, various fishes have adopted two main pathways of nitrogen elimination leading to the production of ammonia on one hand and the synthesis of urea on the other. One ultimate source of nitrogen in both these cases is presumed to be the amide and amine groups of amino acids (Forster and Goldstein 1969). Thus the major route of ammonia formation is by the process of deamination of amino acids via the system of transamination. This pathway couples the transamination of various L-amino acids with α-ketoglutarate to form L-glutamate which is subsequently deaminated by L-glutamate dehydrogenase (Forster and Goldstein 1969; Watts and Watts 1974).

Smith (1929) suggested that branchially secreted ammonia was derived by diffusion directly from blood ammonia preformed in other tissues of the body and delivered as such to the gills. Observations made in the carp, Cyprinus carpio L., by measuring the ammonia levels of the afferent and efferent branchial blood led Pequin (1962) to conclude that all the ammonia excreted at the gills was extracted from blood and that ammonia was not produced in the gill itself. In their later studies (Pequin and Serfaty, 1963), analysis of ammonia in blood returning to the heart via the various main veins suggested that the liver contributed two thirds of blood ammonia and the kidney approximately one third of the total.

Evaluating the relative importance of branchial extraction of preformed ammonia on the one hand and its formation *de novo* at the gills on the other hand Goldstein *et al.*,(1964), found that in the marine teleost Myoxocephalus scorpius, only 60% of the

excreted ammonia was accountable for as preformed ammonia in the blood and that the remaining 40% appeared to come from the enzymatic extraction of α-amino acids-N at the gills.

Although glutaminase is found in the gill tissue,the lack of a net extraction of glutamine from plasma passing through the gills indicates that unlike in the mammalian kidney glutamine doesnot play a role in ammonia formation by gills (Forster and Goldstein,1969).

However, recent work by Payan and Matty (1975) has estimated the contribution of gills to the branchially excreted ammonia at 20%, whereas Cameron and Heisler (1983), have estimated 5-8% as the contribution by the gill metabolism to the net excretion of ammonia.

Makarewicz and Zydowo (1962) have suggested a possible pathway of amino acid deamination involving AMP deamination. In the branchial tissues of several teleost fish and in carp they found a much higher activity of adenosine monophosphate aminohydrolase than that of glutamine and it was suggested that AMP deaminase could be regarded as an important enzyme of nitrogen metabolism. This pathway involves amination of inosine monophosphate, by aspartate and then subsequent deamination of the product, adenosine monophosphate by AMP-deaminase (Forster and Goldstein 1969; Watts and Watts 1974).

Ammonia is also produced by the deamination of adenylates in fish muscle (Driedzic and Hochachka 1976) and gill tissue (Payan 1978). However, the quantitative importance of muscle aminogenesis seems to depend upon the activity of the fish and

the environmental situations (Randall and Wright 1987).

Forster and Goldstein (1969), have also indicated that the relative importance of the sources of blood ammonia probably varies between species and the physiological or environmental status of the fish.

AMMONIA EXCRETION IN FISH

Three basic strategies have been developed by various members of the animal kingdom to excrete ammonia. Most aquatic animals and the teleost fish excrete ammonia directly into their plentiful aquatic environment and are called ammoniotelic animals. However the non aquatic terrestrial animals have developed nitrogen excretion mechanisms that involve the formation of more complex nitrogen compounds such as urea (ureotelic animals) and uric acid (urecotelic animals).In teleost fish, nitrogen is excreted mainly in the form of ammonia, and the gills are the major site of ammonia excretion (Smith 1929). According to Maetz (1972) 98% of the ammonia excreted is eliminated through gills and only minor quantities may be eliminated by the kidneys.

Three possible mechanisms of ammonia excretion have been proposed in fish (Randall and Wright 1987) and they include;

a. passive ammonia flux
b. ionic exchange of NH_4^+ for Na^+
c. passive NH_4^+

Of these, the most significant branchial ammonia excretion mechanism seems to be that of passive diffusion of NH_3.

Since the early works of Jacobs (1940) demonstrating the movement of weak acids and bases across the cell membranes, it has been assumed that ammonia diffuses out passively and freely across the biological membranes, mainly in its neutral, nonionized lipid soluble form down the ammonia gradient. Changes in the NH_3 partial pressure gradient have also been positively correlated with changes in net ammonium excretion in fresh water catfish (Kormanik and Cameron, 1981) and in rainbow trout (Cameron and Heisler, 1983).

The existence of an ammonium ion (NH_4^+) exchange mechanism in fish strongly coupled with the movement of other ions has been known for a long time. The early studies of Korgh (1939) suggested that the branchial excretion of ammonia was linked to cation absorption in fresh water fish. Later, several investigators have examined the details of this exchange mechanism and strong evidence has been provided for the existence of an exchange mechanism in gills of fish involving Na^+ uptake and NH_4^+ excretion (Maetz and Garcia-Romeu, 1964; Payan, 1978; Evans, 1980).

Discussing the alternative pathways of ammonia excretion under high ambient ammonia concentration where the excretion of ammonia along the ammonia concentration gradient fails, Colt and Armstrong (1981) suggested the involvement of this active monovalent cation-exchange transport system involving Na^+ and NH_4^+ for the excretion of ammonia. This system of ammonia excretion has also been described in rainbow trout by Cameron and Heisler (1983), and it seems to be important in minimizing the effect of influxing NH_3 from the external environment, by unloading it in exchange for Na^+, against the gradient and into

the environment (Tucker, 1985).

Another possibility of ammonia excretion by way of passive movement of (ammonium ion) NH_4^+ down its electrochemical gradient, has been indicated (Caliborne, Evans,and Goldstein,1982; Goldstein, Caliborne and Evans,1982). Because NH_4^+ can pass through the lipid cell membrane only to a limited extent and the respiratory epithelial cells of fresh water fishes are joined by tight junctions it appears unlikely that an NH_4^+ diffusion system is of any quantitative importance (Kormanik and Cameron 1981; Randall and Wright 1987). Therefore this system may be only of minor significance.Stewart (1984) has suggested the involvement of Na-K activated ATPase in this method of excretion in the case of rainbow trout Salmo gairdneri under sublethal ammonia toxicity.

CHEMICAL NATURE OF AMMONIA

Ammonia belongs to a class of substances called weak acids or bases, sometimes referred to as weak electrolytes. Weak electrolytes exist in part as undissociated molecules. In all cases the extent to which they are ionized is dependent upon pH and temperature that determines the dissociation constant of these substances.

Ammonia is extremely soluble in water and aqueous solutions and exists in both unionized (NH_3) and ionized forms, as described by the reaction:

$$NH_3 + H_2O \equiv NH_4^+ + OH^-$$

Unionized ammonia is a weak base and depending on the acidity of the aqueous solution binds a hydrogen ion (H^+) to form an ammonium ion (NH_4^+). Conversely the ammonium ion is a weak acid, which depending on the alkalinity of the medium dissociates a hydrogen ion to form the base, ammonia (NH_3) (Visek, 1968).

The ammonia reaction rate in water is extremely rapid with interconversion of NH_4^+ to NH_3 having a half time of less than 50 mSec (Stumm and Morgan, 1981). The dissociation equilibrium of ammonia in aqueous solution (*i.e.* the relative concentrations of the two forms of ammonia) is highly dependent upon pH, to a lesser extent upon temperature and ionic strength (Emerson, Russo, Lund and Thurston, 1975).

For instance, an increase in the pH value of 0.3 units from 7.0 to 7.3 would double the concentration of unionized ammonia (NH_3) in an ammonia solution due to a shift in the equilibrium towards the NH_3 species, although this effect becomes less above pH 8.5 (Alabaster and Lloyd,1980).

The relative concentrations of the two forms of ammonia is also dependent upon temperature, as described by the following expression from Emerson *et al.* (1975) for temperature dependence of the dissociation constant:

$$Pka = 0.09018 + 2729.92 / (273.2+T)$$

where T is the temperature in degrees centigrade (*i.e.*°C).

Thus, a rise in temperature of 10°C doubles the concentration of unionized ammonia (NH_3) present in an ammonia solution over

the temperature range of 0-30°C. The influence of ionic strength on the other hand is such that it results in a decrease in the NH_3 concentration as the ionic strength increases in hard water and dilute saline water.

In most natural fresh water systems containing even up to 200-300 mgl^{-1} dissolved solids, the reduction in percent unionized ammonia attributable to dissolved solids is negligible (Emerson *et al.*1975). However, according to Alabaster and Lloyd (1980), with increasing ionic strength the proportion of unionized ammonia decreases over that in distilled water, being about 10% in water having a hardness of about 250 mgl^{-1}, expressed as calcium carbonate and about 25% in sea water.

Weak acids and bases diffuse across biological membranes in their lipid soluble nonionized forms, which accounts for their ready movement in and out of all cells, plants and animals alike (Jacobs 1940). Similarly the nonionized free ammonia by virtue of its lipid solubility and lack of charge is capable of readily diffusing across the cell membranes, apparently requiring no active transport. Whereas the ammonium ion which is larger (molecular weight of NH_3 = 17, molecular weight of NH_4^+ = 18), hydrated and charged, has very low lipid solubility and cannot readily pass through the charge-lined micropores of the hydrophobic cell membrane components (Jacobs, 1940; Pitts, 1964; Visek, 1968).

Based on experiments conducted in higher animals it has been found that ammonia (NH_3) is much more soluble than CO_2 in water and lipid, and is considerably more soluble in plasma than water, probably because of its lipophilicity. It has also been

estimated from rapid mixing experiments that ammonia concentrations approach equilibrium across the red blood cell membrane in approximately 100mS and the ammonia diffuses significantly less well across the blood brain barrier (for references see Cooper and Plum, 1987). From the available information on fish it is also been known that ammonia is slightly more soluble in fish plasma than in water (Cameron and Heisler, 1983). Studies by Ogata and Arai(1985), have shown that the ammonia concentrations in the erythrocytes of four different fresh water fishes were always higher than those in the plasma.

METHODS OF AMMONIA DETERMINATION

Ammonia in water always exists in its unionized (NH_3) and ionized (NH_4^+) states and this dissociation of ammonia in water is particularly dependent upon pH and temperature. Since it is unionized ammonia that is considered to be toxic and hence more important to fish, the present trend in ammonia toxicology is to express the ammonia levels in terms of its toxic component, unionized ammonia. Although there are no methods developed so far to measure unionized ammonia directly (Thruston, Russo and Smith 1979), more refined ways of calculating the unionized ammonia levels from the measured total ammonia value based on its dissociation chemistry at known pH and temperature values have been made available (Trussel, 1972; Emerson *et al.*, 1975).

For the measurement of total ammonia several methods have been developed both in fresh water and sea water. However, a large number of methods have been adopted for the analysis of fresh water ammonia, but the choice of a method depends on factors

such as the precision, reproducibility, interferences, and its simplicity. The various methods that have been adopted fall into three broad categories as follows.

1. Colorimetric techniques
2. Titrimetric/colorimetric/ionising electrode methods followed by distillation.
3. Ammonia ion sensing electrode method.

The ammonia ion sensing electrode method demands strict temperature control of the sample and standards, apart from requiring both to contain the same level of dissolved species at the time of measurement. Constant stirring of the sample is also necessary. Since the measurement depends on raising the sample pH above 11 to convert all the ammonia ($NH_3aq + NH_4$) to NH_3 (aq), a strict regulation of the diffusion time is necessary for the measurements to avoid ammonia being lost from the sample. Though this method has been recommended for in situ ammonia measurements in field conditions (Stirling 1985) the above said requirements may limit such a use for this method.

The distillation techniques not only require specialized equipment for distillation, the methods are generally unsatisfactory due the possible hydrolysis of organic nitrogenous materials to ammonia (Harwood and Khun,1979). Turbidity and substances such as ketones, amines and aldehydes are also known to interfere with the results.

Direct Colorimetric methods are relatively simpler requiring a minimum of apparatus. A suitable spectrometer or a simple filter colorimeter can be used. This equipment being optically very

efficient, the methods have the advantage of reproducibility. Because of their simplicity and obvious advantages colorimetric methods are often a choice over the distillation and ion sensing electrode methods.

Of the two colorimetric methods traditionally used, the Nesslezaration method is sensitive to numerous variables and is not suited for direct routine application (Harwood and Khun 1970). The method is not sufficiently sensitive for low levels of ammonia and the results are less reproducible. The age of the reagents also affects the sensitivity. On the other hand the phenol hypochlorite method has been described as the most sensitive method, and is best suited for routine analysis due to its simplicity (Harwood & Khun, 1970). The analysis is highly specific and neither urea, creatine or amino acids interfere with the reaction.

In the present study therefore, for total ammonia (NH_3 + NH_4^+-N) determination the phenol hypochlorite method of Scheiner (1976) as outlined by Golterman *et al.*, (1979) was adopted. Although numerous variations to the phenol hypochlorite method exist, (APHA, 1980; Harwood & Khun, 1969; Mackereth, 1978), the method basically consists of reacting a sample containing ammonia with phenol and alkaline hypochlorite to give indophenol blue. The presence of the catalyst sodium nitroprusside intensifies the blue colour at room temperature. The colour intensity is proportional to the ammonia content of the water and is measured spectrophotometrically against a reagent blank.

In the present experiments the colour absorbance was measured with 1cm light path glass cuvettes on a UNIKOV 810 double beam

spectrophotometer equipped with digital display (Kontron Instruments Ltd, U.K.). All absorbance measurements were made at 635nm. At the time of each estimation a series of standards were prepared and a standard curve was constructed and the sample ammonia values were calculated, using a computerised programme.

Unionized ammonia levels from the measured total ammonia were calculated according to Emerson *et al.*, 1975), with the use of the following formulae, or from their tabulated PKa values.

$$\text{Unionized ammonia}(mgl^{-1}) = \frac{\text{Total ammonia}(mgl^{-1})}{1 + \text{antilog}\,(PKa - pH)}$$

where PKa = (-log Ka) is the negative logarithm of the acid dissociation constant of NH_4^+ ion which could be calculated from the relationship

PKa = 0.09018 + 2729.92/T

Where, T is the Absolute temperature in K, and

K = (C + 273.16), C being the measured water temperature in degree centigrade.

The pH and temperature values used in the unionized ammonia calculations were the measured mean values from each treatment.

LOSS OF AMMONIA FROM WATER

Although various processes have been known to result in the build up of ammonia both in natural waters and in culture conditions, the processes by which ammonia can be lost from the

water have been found to be limited.

Tucker (1985), while discussing the dynamics of ammonia in catfish ponds, has stated that currently there is no practical method to reduce either total ammonia or unionized ammonia concentrations in large commercial channel catfish ponds, other than the exchange of water; although the concentration of unionized ammonia can be decreased temporarily by decreasing the pH of the water by adding an acid forming substance which may only be practicable in a small scale.

Vamos and Tasnadi (1967) have recommended the use of $CuSO_4$ to reduce the ammonia levels in carp ponds and reported that the resulting cupro ammonium compound is non toxic to fish. Herbert and Van Dyke (1964) however, found that they cause joint toxicity.

Apart from these limited ways of controlling ammonia in natural water such as ponds and lakes, various other processes have been found to be involved in the ammonia removal process from water.

Three possible routes of ammonia loss have been known to operate and are:

1. Losses due to nitrification, 2. Ammonia volatilization and 3. Biological assimilation by phytoplankton and blue green algae.As ammonia is an intermediate substance of nitrogen cycling in natural waters, it is oxidized to less toxic nitrates by the process of nitrification.

Much of the ammonia lost in fish culture facilities, especially those of recirculating systems with well established biofilters,

be accounted for solely due to nitrification processes. However such losses in other fish culture facilities and natural water bodies may be less significant.

Nitrification is the process of biological oxidation of ammonia to nitrite and nitrate by autotrophic bacteria in aerobic conditions. Nitrosomonas sp and Nitrobactor are the principal nitrifying bacteria in fish culture system (Spotte, 1970) as indicated below.

$$NH_4^+ + OH^- + 1.5O_2 \xrightarrow{\text{Nitrosomonas spp}} H^+ + NO_2^- + 2H_2O$$

$$NO_2 + 0.5\ O_2 \xrightarrow{\text{Nitrobactor spp}} NO_3$$

Tucker (1985), based on his observations on the dynamics of ammonia in catfish ponds, suggested that up to about 57% of nitrogen in the ponds was lost by the process of nitrification and volatilization.

Under high pH conditions (pH = 9.0 and above) ammonia has been found to volatize and escape into the environment. Murphy and Brownlee (1981) have estimated the volatilization rates of ammonia at 15-30$\mu g l^{-1}$/hr during periods of high pH, high ammonia, and strong breezes in shallow hypertrophic Canadian lakes.

Aquatic plants, particularly phytoplankton and blue green algae , are known to extract ammonia fairly rapidly from water as a nitrogen source accounting for considerable ammonia losses in water (Murphy and Brownlee 1981a 1981b ; Weiler 1979).

It has also been found in catfish ponds that the losses of ammonia due to phytoplankton assimilation are considerable (Tucker 1985), and it was suggested that this biological assimilation process by phytoplankton, in combination with volatilization processes, was responsible for the maintenance of low total ammonia nitrogen values in catfish ponds.

The processes of volatilization and phytoplankton assimilation may be of no importance in intensive hatchery rearing conditions, where conditions such as high phytoplankton densities and elevated pH values very uncommon.

Gerking (1955), investigating the possibilities that ammonia might volatilize under experimental conditions from well aerated water, within a pH range of 7.39 to 8.91, and using NH_4Cl as a source of ammonia, found no ammonia loss due to volatilization. Similar results were noted by Sheehan and Lewis (1980).

TOXICITY OF AMMONIA TO FISH

The toxicity of ammonia to fish has been extensively investigated in the past 30 years. Detailed reviews on ammonia toxicity to fish have been provided, (EIFAC, 1970; Alabaster and Lloyd, 1980 ; Haywood, 1983 ; Meade, 1985) wherein various aspects of ammonia toxicity are considered in detail.

The toxic action of ammonia to fish has been classically attributed to unionized ammonia (Whurman and Wolker, 1948; Downing and Merkens, 1955; Vamos and Tasnadi, 1967) and the ionized ammonia is considered as non toxic or relatively less toxic. Various environmental factors have been known to affect

the toxicity of ammonia in fish, such as pH, temperature, carbon dioxide, dissolved oxygen, water hardness, alkalinity and salinity. Apart from these, a few biological factors such as previous acclimation of fish to ammonia, size and sex of the fish have also been known to alter the toxicity of ammonia in different fish species. Hence consideration of these factors and their role in influencing ammonia toxicity are important.

Generally a reduction in the pH value of a solution results in a shift in the NH_3/NH_4^+ equilibrium towards the ammonium ion, resulting in a reduction in the toxic unionized ammonia fraction. On the other hand a rise in the pH value results in an increase in the unionized ammonia concentration and decrease in the ammonium ion concentration.

Thus the percent unionized ammonia of a solution depends upon the pH of the solution as indicated by the relationship

$$\% \text{ UIA} = \frac{100}{1 + \text{antilog (PKa - pH)}}$$

In fact the experimental basis that unionized ammonia is toxic and ionized ammonia is less toxic was based on the influence of pH upon the NH_3/NH_4^+ equilibrium. Thus the experimental observations on fish of Whurman, Zehender and Wolker (1947), confirmed the earlier opinion that ammonia is more toxic in an alkaline medium than in an acid medium. Further experimental confirmation that the concentration of unionized ammonia and, in turn, the toxicity of ammonia is dependent up on the pH value, came from the observations of Downing and Merkens (1955). Downing and Merkens reported that at pH 7.0 the concentration

of ammonium chloride required to give three different concentrations of unionized ammonia were approximately 10 times greater than those required at pH 8.0.

However, more recent studies (Armstrong , Chippendale, Knight and Colt, 1978; Thurston, Russo, and Vinogradov, 1981; Goldstein, *et al.* 1982; Sheehan and Lewis, 1986) have indicated a more significant role pH in the toxicity of ammonia than that of mere by controlling the NH_3/NH_4^+ equilibrium, indicating a toxic action of NH_4^+ or an increased susceptibility of fish to ammonia under low pH.

It is argued that, if the unionized ammonia was solely responsible for the toxic action on the given test fish, then one would expect that the LC50 values in terms of unionized ammonia would be reasonably constant for all test fish of a single species, regardless of the pH of the solution and the total ammonia concentration present. Such was not the case in certain other studies (Thurston, Russo,and Vinogradov 1981, Sheehan and Lewis 1986). The LC50 values obtained by Thurston *et al.* (1981), for UIA were markedly less at pH 6.5 than at pH 8.0 and pH 8.5. Therefore it was strongly suggested that the toxicity of unionized ammonia over the pH range 6.5 to 8.5, which is considered as an acceptable range for fresh water aquatic life, is constant in that the increased pH value doesnot increase the toxicity of ammonia, and that NH_4^+ ion exerts a toxic effect under low pH and high ammonia concentrations.Similar increase, in ammonia toxicity with decreasing pH values have been demonstrated in channel catfish, I. punctatus by Sheehan and Lewis (1986).

Colt and Armstrong (1981) suggested that at low pH, where almost all the external ammonia present in water exists in its ionized form (NH_4^+), the alternative ammonia excretion mechanism involving Na^+ uptake and ammonium excretion is affected. This therefore implicated an inhibition of Na^+ influx as the possible significant contributing factor to the toxicity of ammonia at low pH.

As indicated already, the toxicity of ammonia increases with rising temperature owing to the increase in the proportion of unionized ammonia within a given ammonia solution. A rise in temperature of 10°C is known to double the concentration of UIA present in a solution (Alabaster and Lloyd, 1980) and hence the toxicity of ammonia to fish to a similar magnitude. However, the temperature ranges beyond the optimal tolerance limits of a fish species, either low or high, may in itself exert some detrimental effect in addition to ammonia.

Burrows (1964) in the case of chinook salmon (O.tsawytcha) and Brown (1968) in the case of rainbow trout have shown that at very low temperatures unionized ammonia became more toxic contrary to the normal trend.

Thurston and Russo (1983) found that temperature increase over the range of 10 to 19°C reduced the toxicity of ammonia to rainbow trout. Their results were in agreement with those of The Ministry of Technology (1968) who reported that the toxicity of NH_3 to both juvenile and adult rainbow trout was much greater at 5°C than at 18°C.

Although free carbon dioxide is not an important aquatic

pollutant in natural waters (Lloyd and Shift, 1976), an increase in the level of metabolic carbon dioxide offsets the toxicity of ammonia, by decreasing the pH value of the water, until a concentration of free carbon dioxide was reached which was itself toxic to fish (Alabaster and Herbert, 1954).

Based on their later studies (Lloyd and Herbert, 1960) proposed that it was not the pH value of the bulk of the water which was important in determining the toxicity of ammonia but the pH value of the water at the gill surface. If the concentration of the free carbon dioxide in the water is very low the amount excreted by the fish will considerably reduce the pH value at the gill surface, but the extent of this pH change will become less as the level of free carbon dioxide rises in the bulk of the water.

A reduction in the dissolved oxygen level of water usually results in an increase in the toxicity of ammonia (Downing and Merkens, 1955; Vamos and Tasnadi, 1967; Alabaster *et al.*, 1979, Thurston *et al.*, 1981), but in the case of fat head minnows Pimephals promelas. Thurston, Russo and Phillips (1983) did not find any relationship between ammonia toxicity and dissolved oxygen concentrations over the range of 3 to 9 mgl^{-1} dissolved oxygen. This deviation in the results was attributed to a possible species specific difference although, they did not rule out possible causes due to experimental methods by some earlier workers.

Variations in water hardness reportedly does not seem to have any effect on the toxicity of ammonia to minnows and rainbow trout (Whurman and Wolker, 1953; Herbert, 1961; both cited by

Alabaster and Lloyd 1980). However, Alabaster and Lloyd (1980) themselves have indicated that the proportion of UIA decreases with ionic strength; the decrease over that in distilled water is about 10% in a water having a hardness of about 250 mgl^{-1} UIA, expressed as calcium carbonate. However, Emerson *et al.*, (1975), indicated that although there is a decrease in the NH_3 concentration as the ionic strength increases in hard water, in most natural water systems even up to 200-300 mgl^{-1} dissolved solids, the reduction in the percentage NH_3 attributable to dissolved solids is negligible.

Dilute saline water seems to have a beneficial effect on the toxicity of ammonia to salmonids. In rainbow trout it has been shown that the toxicity of ammonia decreases with increasing salinity up to 30% sea water (= 10.5 ppt) (a concentration approximately isotonic with the fish blood), but above that toxicity increases up to 100% sea water (=35 ppt) (Herbert and Shruben, 1965). Sousa, Meade and Wolker (1974) have also similarly demonstrated, in chinook salmon (O. tshawytsha) that fish could tolerate higher concentrations of ammonia at 12 ppt salinity than they could at either 6 ppt salinity or in fresh water.

Alkalinity of the water does not seem to have any effect on the toxicity of ammonia, except that it may play a part in determining the pH value of the water in conjunction with the level of free carbon dioxide (Alabaster and Lloyd, 1980).

Prior acclimation of fish to sublethal levels of ammonia has been known to increase their resistance to lethal concentration in their subsequent exposure. This has been shown in carp

(Vamos, 1963) and in trout (Lloyd and Orr, 1969; Schulze-Wiehenbrauck, 1976; Thurston *et al.* 1983). Redner and Stickney (1979), reported that tilapia (Tilapia aurea) exposed to sublethal concentrations of unionized ammonia at 0.43-0.53 mg^{-1} for 35 days could tolerate as high as 3.4 mg^{-1} UIA without any mortalities for 48 hours whereas, in the case of nonacclimated fish the 48 hrs LC50 were only 2.40 mg^{-1} UIA.

Rainbow trout and cutthroat trout subjected to fluctuating concentrations of ammonia at levels below those of acutely toxic concentrations were subsequently better able to withstand exposure to higher fluctuating concentrations than fish not previously so acclimated.

Variations in relative susceptibility has also been demonstrated to be dependent upon the age of the fish in several cases. Rice and Stokes (1975) noted that the eggs and alevins of rainbow trout, before the yolk absorption stage, were about 50 times more resistant to unionized ammonia than they were after the yolk absorption stage and the adult stage. Similarly, Reichenbach-Klinke (1967) found that the fry of rainbow trout were more sensitive to ammonia than the larger trout. Calamari, Marchetti and Vailati (1981), have shown that the developmental stages of rainbow trout from eggs to hatching were more resistant to unionized ammonia values of greater than 0.480 mgl^{-1} whereas the 70 day old fry were sensitive to only 0.160 mgl^{-1} UIA, but later at the fingerling stage became resistant to 0.440 mgl^{-1} UIA. According to Thurston, Russo and Phillips (1983) the toxicity of ammonia to fat head minnows, Pimephales promelas was not related either to size or source of the fish used in their experiments.

Hemens (1966) reported a greater resistance of female mosquito fish, Gambusia affinis, to total ammonia poisoning than that shown by the males of the species. However the size difference had no apparent effect on susceptibility.

The toxicity of ammonia to fish is dose dependent and accordingly it could be acutely toxic or subacutely or chronically toxic. Acute toxicity is usually lethal and chronic toxicity may be lethal or sublethal (Sprague, 1973). Excessively high ammonia concentrations that are lethal and result in death within a specified period of 24, 48 or 96 hours are referred to as acutely toxic levels in general. Such acute toxic levels are demonstrated by measuring the LC50's (lethal concentrations required to kill 50% of the experimental fish) for comparative purposes. While the broad physiological mechanism of toxic ammonia effects are probably generally similar in most types of fish, the actual toxic thresholds may vary quite widely in different genera, particularly since there are marked differences in nitrogen excretory metabolism and ammonia detoxification in crustacea, elasmobranchs and teleosts (Hampson,1976). There is considerable variation between different groups or genera of fish to such a magnitude that a sublethal ammonia concentration of one fish species of a particular group may constitute an acutely lethal dose to another species of a different group. From the table 2.1 which provides a comparitive account of the reported LC50 values between the more sucecptiable and more resistant fish species such a difference is more obviously indicated.

Table 2.1. A comparison of the reported toxicity values of unionised (UIA) to different fish species.

Species	Size	Lethal concentration of UIA mgl^{-1}	Author
Rainbow trout *Salmo gairdneri*	40g (23-53)	0.41 (48-h LC50)	Ball (1967)
	9-12g	0.86-0.94 (96-h LC50)	Thurston & Russo 1983
Cut-throat trout *Salmo clarki*	1-3g	0.5-0.8 (96-h LC50)	Thurston *et al.* (1978)
Common carp *Cyprinus carpio*	0.3g	1.7 (96-h LC50)	Hasan & Macintosh(1986)
	24-43g	0.91-1.56 (48-h LC50)	Dabrowska & Sikora (1986)
Tilapia *Oreochromis aerues*	7-9cm	2.4 (48-h LC50)	Redner & Stickney (1979)
Fathead minnows *Pimephales promelas*	0.22-1.7g	1.52-2.83 (96-h LC50)	Thurston *et al* (1983)
Channel catfish *Ictalurus punctatus*	20-47g	2.36 (16-h LC50)	Robinette (1976)
	7-13cm	1.39-1.82 (24-h LC50)	Tomasso *et al* (1980)

The mechanism of ammonia toxicity is thought to be possibly by "mass law" prevention or reversal of normal nitrogen metabolism tcv that is, it is caused by high ammonia concentrations in the blood resulting from prevention of excretion of ammonia, or even its uptake from water at the surface membranes, particularly at gills (Hampson,1976).

Like other higher animals acute lethal toxicity of ammonia to fish has been known to cause increased ventilation, hyperexcitability, convulsions, and coma followed by death (Smart,1975; Daoust and Ferguson 1984). Similar to higher animals such changes are readily reversible and the fish can revive and become normal if they are transferred to ammonia free water before 95% of the average survival time in that particular univtconized ammonia concentration has elapsed (Smart, 1975).

The exact mechanism as to how ammonia brings about death under high concentrations has still not been wholly explained. However, from the extensive works available on toxicity of ammonia in higher animals it has been implicated that high ammonia levels bring about changes in the energy metabolism of the brain and structural changes in brain cells (Visek, 1968; Walker and Schenker, 1970).

Cooper and Plum (1987) have extensively reviewed the literature on the biochemicavl and physiological aspects of brain ammonia and discussed various aspects of possible mechanisms of ammonia neurotoxicity. Ammonia has been suggested to interfere in various processes such as;

1.Electrophysiological properties of neural tissues.

2.Interfere with neurotransmitter function.

3.Bring about morphological changes in astrocytes and neurons
4.Interfere into biochemical pathways such as carbohydrate.
5.Fat and protein metabolism in various tissues of the body.
6.Alter brain energy metabolism.
7.and Alter glycolysis and tricarboxylic acid cycles in brain.
Cooper and Plum (1987) summarized that the glutamine synthetase of the astrocytes forms an enzymatic barrier that effectively converts both cerebrospinal derived and blood derived ammonia to glutamine under normal circumstances. Under high ammonia situations the steady state ammonia levels are raised proportionately, thereby saturating the "enzymatic detoxifier" of the astrocytes and impeding the capacity of the brain to protect itself against additional ammonia surges, thereby contributing to neurological dysfunction. Excessive ammonia interferes with brain energy metabolism possibly by inhibiting discrete steps of the tricarboxylic acid cycle and in part by interfering with the malate aspartate shuttle.

The acute toxicity of ammonia in fish is less well understood. Early workers suggested that ammonia may impair gas exchange (Brokway 1950) and subsequent workers indicated severe gill damage as the cause of death (Burrows, 1964; Flis, 1968a,1968b).

Smart (1975), investigating the possibility that acute ammonia may bring about severe gill damage in rainbow trout found relatively minor histopathological changes (in acute toxic studies lasting for 2 to 5.5 hours) compared to prolonged sublethal experiments. Further studies by Smart (1975) indicated that although a fall in dorsal aortic blood oxygen pressure was noticed, the oxygen uptake by fish had been enhanced due to hyper ventilation, and therefore he rejected the theory that the

gill damage and reduced oxygen exchange was the primary cause of the toxic action of ammonia. Based on his observations of the fishes behaviour such as hyperexcitability, coma, convulsions, hyperventilation and the fact that the ready reversibility of these changes was very similar to those described in mammals, Smart suggested that the acute toxic action of unionized ammonia in fish could well be the same as in mammals. Smart (1975) also noted a depletion in high energy phosphates such as adenosine triphosphate (ATP) and phosphocreatine in the brains of rainbow trout and indicated a possible impairment of cerebral energy metabolism.

Arillo, Margiocco, Melodia, Mensi and Scheaone (1981) measured various metabolites in rainbow trout, involved in the neurochemical alterations typical of ammonia toxicity known in mammals and came to the conclusion that the toxic mechanism in fish is similar to that found in other animals, and as reported by Smart (1975) it affects the energy metabolism of brain.

Under high stocking densities typical of intensive fish culture, sublethal ammonia levels have long been suspected of causing reduced growth, damage to gills and other organs, and have been identified as the predisposing factors of bacterial and infectious diseases.

Several investigators have looked at the effects of ammonia under sublethal and extended lethal concentration levels. Apart from mortalities and growth reductions, varying responses ranging from behaviourial to biochemical, and physiological to pathological changes have been proposed.

destruction of lamellae and lamellar epithelium was extensive. In rudd exposed to sublethal concentrations of ammonia at 0.10 mgl^{-1} UIA for 95 days, histopathological changes were seen only in the epidermis (Department of Environment,1971).

Smith and Piper (1975) exposed rainbow trout to chronic levels of metabolically produced ammonia for 12 months and reported degenerative changes in the gills, livers and in the intestinal mucosa. In fish exposed to low levels of ammonia between 0.013 to 0.025 mgl^{-1} UIA, all tissues were relatively normal except gills, which only exhibited mild hyperplasia, hypertrophy and occasional lamellar fusion. However, in fish subjected to 0.033 mgl^{-1} UIA, a reduction in the splenic lymphoid tissue, mild necrosis of the intestinal mucosa, and total necrosis of liver cells were noted.The gill tissues showed extensive lesions consisting of severe hyperplasia of the gill epithelium, extensive fusion of the lamellae, in addition to occasional fusion of the filaments. Lamellar fusion was quite common at the tip of the filaments and such lamellae were oedematous at the bases. Examination of the oedematous tissue revealed the presence of cellular degeneration and mild inflammatory response. In regions where severe hyperplasia was absent hypertrophy of the gill epithelium was common. Blood filled aneurysms were commonly seen, some of them containing liquified blood in addition to inflammatory and necrotic changes. Liver cell necrosis was characterized by pyknosis.

Sousa, Meade and Wolke (1974) exposed chinook salmon fingerlings to low (1ppm) and high (50ppm) total ammonia concentrations in fresh water and increasing concentrations of sea water up to

12ppt for a period of 20 days at pH 6.5 and temperatures between 14-15°C. Microscopical examination of the gills in both cases did not reveal any pathological changes.

Robinette (1976) exposed channel catfish, Ictalurus punctatus, to various sublethal levels of ammonia and noted a hyperplastic reaction in gill tissue, and suggested that such damage to gill surface would interfere with gill function in oxygen uptake and this may be a contributing factor in growth reduction. Smart (1976), investigating the effects of ammonia on trout gill structure concluded that the gill damage resulting from exposure to acutely lethal concentrations was relatively minor (note: his acute lethal studies lasted for between 2 and 5.5 hours) and thus unlikely to have been a primary cause of death, and suggested an alternate mechanism. However, following a long term exposure of trout to ammonia, he found severe histopathological changes in the gill structure and a high incidence of fungal disease. The histopathological changes noted at sublethal ammonia exposure included thickening of the lamellar epithelium and increased mucus secretion. Lamellar telangiectasis described as haematomas were a characteristic feature noted by this author, similar to those of Bullock (1972) and Smith and Piper (1975). However, Smart (1976) did not observe gill hyperplasia as noted by others. Redner and Stickney (1979) noted capillary congestion, haemorrhaging and telangiectasis in Tilapia aurea exposed to both acutely lethal ammonia concentrations for 24 hrs and sublethal ammonia concentrations for 35 days. In the cutthroat trout, Salmo clarkii, exposed to 0.34 mgl^{-1} unionized ammonia for 29 days, degenerative changes in the gills, kidney and liver tissues were noticed by Thurston, Russo and Smith (1978). Gill changes

included hypertrophy of the epithelium, necrosis of epithelial cells and separation of the epithelium due to oedema. In the kidneys mild hydropic degeneration was noted along with accumulation of hyaline droplets in renal tubule epithelium. These changes in the kidneys were interpreted as probably related to an increase in the permeability of the fish tissues to water and an increased urine output, and the hyaline droplets due to reabsorption of excessive amounts of proteins from the glomerular filtration.

In the developing stages of rainbow trout, from egg to larvae, subjected to sublethal ammonia concentrations for 25 days, Calamari, Marchetti and Vailati (1981), noted both macroscopic and microscopic alterations. The macroscopic alterations included structural malformations such as bending of the body axis in spiral shapes and duplication of heads described as "duplicitas anterior", whereas the histological microscopical observations included changes in the epidermal thickness and an increase in the mucous cell numbers and size. The pronephros showed vacuolation and contained accumulations of eosinophilic droplets, while the eyes and retinas were incompletely developed. Changes in the nerve tissue and neuronal tube were obviously evident from the excrescences found in the encephalon. The frequency of the microscopical alterations were correlated to ammonia concentrations above 0.025 mgl^{-1} UIA and 0.063 mgl^{-1} UIA respectively,for epidermis and pronephros microscopical malformations.

In milk fish, Chanos chanos, subjected to acutely lethal concentrations of ammonia for 24, 48, 72 and 96 hours at 20.65 mgl^{-1} total ammonia, Cruz and Enriquez (1982) found severe

histological changes essentially similar in all cases. The changes included epithelial hypertrophy found all along the length of the lamellae, mild lamellar and basilamellar hyperplasia, along with epithelial lifting and telangiectasis. Other changes such as vacuolation of the lamellae, increased mitotic activity in the gill epithelium and vacuolations in the blood cells were also noted.

Soderberg, McGee, Grizzle and Boyd (1984) and Soderberg (1985) found varying gill lesions in rainbow trout and channel catfish under pond rearing conditions with fluctuating ammonia concentrations of 2.3 to 8.4 μgl^{-1} UIA over the season under study (61 days period) and with a daily maximum fluctuation range of 8.3 to 48.7 μgl^{-1} UIA. Lesions found in the gills included hyperplasia, oedema and telangiectasis and were attributed to the fluctuating environmental ammonia, even though the average exposure levels were low.

Klontz, Stewart and Eib (1985) reported severe alterations in gill tissues of rainbow trout subjected to both constant and intermittently exposed unionized ammonia concentrations for prolonged periods of time. Lamellar hypertrophy was noticed in fish constantly exposed to 0.03 mgl^{-1} UIA and in intermittently exposed fish at 0.05 mgl^{-1} UIA after 2 weeks later resulting in the separation of lamellar epithelium termed by these authors as epithelio capillary separation (ECS). These changes increased in their frequency by week 4, and after weeks 8-12 up to 40% of the lamellae at 0.03 mgl^{-1} UIA constant exposure and up to 70% at 0.05 mgl^{-1} UIA constant exposure exhibited ECS. In case of fish intermittently exposed 0.05 mgl^{-1} UIA, 30% of lamellae were affected by the end of 12 weeks, whereas the untreated fish

exhibited no such changes. The pathological alterations were highly correlated to high densities of the fish and ammonia.

In one of the longest experiments conducted so far on rainbow trout, extending for 5 years, and involving adult parental rainbow trout and their F1 and F2 generations under chronic ammonia concentrations, Thurston, Russo, Luedtke, Smith, Meyn,Chakoumakos,Wang and Brown(1984) have further demonstrated histopathological lesions in gills, kidneys, livers and in the epidermis. These authors stated that the gill lesions found in their study were typical of those found by other authors (Burrows 1964; Flis 1968a,b; Smart 1975; Smith and Piper 1975) and included histological changes such as hypertrophy of the gill lamellae with accompanying basal hyperplasia, lamellar epithelial separation, epithelial necrosis, aneurysms and mild to moderate fusion of the lamellae. The histopathological changes were apparent in gills exposed to 0.013 to 0.073 mgl^{-1} UIA for periods of 4, 7 and 11 months.

However, Thurston, Russo, Meyn, and Zajdel (1986) exposed fathead minnows for a period of one year under chronic ammonia levels of 0.07 to 0.96 mgl^{-1} UIA, but did not find any gill lesions. Similar results were reported by Smith (1984) in the same fish species, instead extensive brain lesions were found. These lesions appeared as swollen, darkened areas on several fish heads at 0.21 mgl^{-1} UIA and higher. At 0.42 mgl^{-1} UIA some of these growths were so massive that they replaced the eyes of the fish.

Recent experiments by Lang, Peters, Hoffmann and Meyer (1987), have further noted the changes such as increased ventilation,

increased mucous cells in the skin epidermis, and slight but significant gill tissue proliferations (hyperplasia) in the gill tissues of rainbow trout exposed to sublethal ammonia concentrations of 0.025 mgl^{-1} UIA for 4 weeks.

Although the selected literature reviewed here on the toxicity of ammonia to various fish tissues and organs indicates strongly the deleterious effects of ammonia, in spite of the lesions being inconsistently present, two of the latest reports on ammonia, toxicity have directly contradicted previous results.

Mitchell and Cech (1983) investigating the possibility that ammonia causes characteristic gill hyperplasia in channel catfish failed to confirm histologically that ammonia alone was the direct cause of gill hyperplasia. Instead they implicated low levels of residual chlorine compounds (monochloramines) typically found in municipal water supplies and charcoal filtered water, together with moderate ammonia levels, as the actual cause of gill hyperplasia. In none of the ammonia exposed fish (at 580 $\pm$ 180 μgl^{-1} UIA for 83 days) could they find any sign of hyperplasia or any other gill damage and insisted that the hyperplasia reported by Robinette (1976) in channel catfish was due to residual chlorine compounds in combination with ammonia.

In their recent investigation on rainbow trout, subjected to unionized ammonia concentrations of 0.3 and 0.4 mgl^{-1} for 90 days Daoust and Ferguson (1984) found no gill lesions and hence declared that ammonia *per se* does not cause any gill lesions in rainbow trout.

These reports have not only caused considerable concern as to the exact role of ammonia at sublethal concentrations, but have at the same time created a confusion as to the present understanding of ammonia toxicity on gill pathology as highlighted by Mallatt (1985) and Meade (1985). A detailed analysis of the literature describing ammonia caused gill lesions also indicates that no attempts have been made to utilize the widely available scanning and electron microscopical techniques to study the effects of this pollutant on fish gills.

The present study was initiated with a view to investigate the toxic effects of ammonia on the gills of two different fish species, simultaneously utilizing scanning and transmission electron microscopical techniques in conjunction with histological methods. Attempts were also made to describe the normal gill structure for common carp where gill structure has not been a subject of either SEM or TEM study in the past, and for the rainbow trout whose gill structure has been extensively investigated, so as to provide comparative and more meaningful information on the ammonia induced gill pathology.

CHAPTER 3

GENERAL MATERIALS AND METHODS

The methods and materials common to all the experiments are presented in this general materials and methods chapter, where as specific aspects of materials and methods applicable to individual experiments are detailed in the appropriate chapters.

3.1. WATER QUALITY (Methods of Measurement of Physico-chemical Parameters)

AMMONIA DETERMINATION

Total ammonia was determined by the phenolhypochlorite method of Scheiner (1976) as outlined by Golterman *et al.* (1979). Here ammonia reacts with phenol and alkaline hypochlorite to give indophenolblue. The blue colour is intensified by the use of a sodium nitroprusside catalyst at room temperature. The colour intensity, which is proportional to the amount of ammonia present is measured photometrically as absorbence at 635 nm against a reagent blank. A series of standards with known concentrations of ammonia were prepared and a standard curve was plotted. From this the sample ammonia concentrations were calculated. Occasionally ammonia concentrations were also analysed simultaneously on a technicon-sampler IV autoanalyser for the purpose of comparison of results with those obtained by the manual method.

NITRITE

Nitrite-nitrogen (NO_2-N) concentrations from the experimental water samples were determined according to the method of Mackereth *et al.*(1978). Nitrite reacts with sulphanilamide and later with N-1-naphthylethylene diamine dihydrochloride to yield a red Azo-dye which was determined spectrophotometrically at 540

nm. Alternatively nitrite was also analysed on the auto-analyser.

NITRATE

Nitrate nitrogen (NO_3-N)analysis was carried out on the auto-analyser technicon-sampler IV. Here the nitrate is first reduced to nitrite by the cadmium copper couple and then the resulting nitrite is determined on the same principle as nitrite analysis mentioned above.

pH (HYDROGEN ION CONCENTRATION)

The pH measurements of the water samples were made with the help of a Philips PW 9409 digital pH meter fitted with a glass combination electrode. Prior to use, the pH meter was calibrated using pH 4.0 and pH 7.0 standard buffer solutions (BDH, UK.).

CONDUCTIVITY

Conductivity was measured with the help of a PHOX-52 conductivity meter and was expressed as $\mu S\ Cm^{-1}$

DISSOLVED OXYGEN

Dissolved oxygen concentrations were measured with a CLANDON YSI model 57 oxygen meter provided with an oxygen sensing electrode and a mechanical stirrer.

TEMPERATURE

Water temperature measurements were made with a Gallenkamp glass mercury thermometer.

TOTAL HARDNESS

Total hardness was measured titrimetrically using standard EDTA solution (Ethylene diamine tetraacetic acid). BDH volumetric EDTA solutions and total hardness indicator tablets, and ammonia buffer were used.

TOTAL ALKALINITY

Total alkalinity was determined according to the method of Mackereth *et al* (1978). The method involves titration of the water sample with standard hydrochloric acid to a pH of 4.5. BDH 4.5 colour indicator was used to give a colour change at this pH. Alternatively the pH was followed using a pH electrode from a pH meter. Total alkalinity was expressed as $meql^{-1}$.

3.2. HISTOLOGICAL TECHNIQUES

TISSUE SAMPLES

All gill holobranchs except two from any one side of each fish used in the experiments, were sampled for light microscopic histological preparations. The operculum was removed and the gill holobranchs were carefully dissected out with the help of fine scissors and fine pointed forceps and fixed immediately in the appropriate fixative solution. Other tissues such as kidney, spleen, liver and pancreatic tissues were also sampled for general light microscopic histological observations. No dead fish were sampled for histological studies.

Since gills were the primary organs of study in the histopathological investigations, they were always sampled first

and as quickly as possible after the fish had been sacrificed. Sampling gills first always resulted in immediate spilling of blood over the gill tissues leading to large scale accumulations of blood cells in the interlamellar and interfilamental areas and sticking of blood cells onto the filamental and lamellar epithelium. To avoid this problem fish tails were first severed which allowed the blood to flow out before the gill was dissected. This procedure greatly reduced the problem of blood cell accumulations on the gill tissue enabling clearer observation of the epithelial surfaces without any obscurity, especially under scanning electron microscopy.

TISSUE FIXATION

All tissues for histological work were fixed in 10% phosphate buffered formalin and left for at least one week before processing. For comparative study, tissues were also occasionally fixed in aqueous Bouin's fluid.

TISSUE PROCESSING

All tissues to be processed were cassetted, labelled and autoprocessed on a histokine (HISTOKINETTE 2000). This involved passing of the tissues through different alcohol grades, followed by absolute alcohol, chloroform and then impregnation in molten wax. Tissues were blocked in suitably sized moulds using molten wax and were cooled rapidly on a cold plate. A detailed processing schedule is provided in appendix 2.

SECTIONING

Tissue blocks were trimmed to bring the tissues to the surface of the block and whenever necessary surface decalcification was carried out by treating the surfaces of the trimmed blocks in a surface decalcifier (RDC-histolab). The blocks were then washed, cooled on a cold plate and 3 to 5µm sections were cut on a Leitz-wetzlar microtome using Richert-Jung disposable microtome blades. Thin sections were floated on a water bath maintained at 40°C and were collected on prewashed wet glass slides. The slides were then marked and dried before they were stained.

STAINING

For general observations all sections were stained with haematoxylin and eosin. Special stains such as periodic-acid and Schiff's (PAS), alcian blue (AB), and combined alcian blue-PAS, were carried out as and when necessary to demonstrate different components of the tissues. Procedures as outlined in Carlton's histological techniques (Drury and Wallington, 1980) were followed for the preparation of stains and staining methods. Stained sections were mounted in synthetic mounting medium (Pertex-histolab). Staining schedules used for different staining processes are detailed in appendix 3,4,5 and 6.

PHOTOMICROGRAPHY

The photomicrographs from the histological sections were taken on Leitz-orthomat automatic photomicroscope. For all black and

white photography Ilford Pan F-135 film was used, where as for colour prints Kodak Ectachrome professional film was used.

3.3. ELECTRON MICROSCOPY

TISSUE FIXATION

Tissue fixation procedures followed were the same for both scanning electron microscopy (SEM) and transmission electron microscopy (TEM). All gill samples (ie 1st and 2nd gill arches from all the fish) were fixed immediately after their dissection in ice cold 2.5% glutaraldehyde in 0.2M cacodylate buffer. After 2 hours fixation at 4°C the tissues were given two washes of 1 hour each in 0.2M buffer. Tissues were then post fixed in cacodylate buffered 1% osmium tetroxide for 2 hours at 4°C and washed twice in the same buffer before they were dehydrated in 70% alcohol. After two changes in 70% alcohol each group of tissues was sub divided into two groups to be processed further separately for SEM and TEM studies. Tissues were stored at this stage in case further processing had to be delayed as recommended (Hayat 1978).

SCANNING ELECTRON MICROSCOPY (SEM)

Gills to be processed for SEM were transferred to 70% acetone from 70% alcohol and were further dehydrated in 2 changes of 100% acetone. Acetone here serves as an intermediate fluid while taking the tissues to critical point drying using liquid carbon dioxide as the transitional fluid. The gills were critical point dried in a Polaron-E 300 critical point drier, mounted on aluminum stubs using colloidal graphite paste and were then coated with gold-palladium in an Edwards S-150 Sputter coater

before they were examined under an ISI-60A scanning electron microscope.

TRANSMISSION ELECTRON MICROSCOPY (TEM)

Tissues to be processed for TEM were transferred from 70% alcohol to 100% alcohol for further dehydration and after two changes in 100% alcohol, were taken in to Epon 812 medium hard resin (TAAB) through different grades of propylene oxide and resin mixtures. After overnight impregnation in fresh resin at room temperature and at 37°C for 3 hours, tissues were embedded in the moulds using fresh resin and were allowed to harden at 60°C for 12 to 24 hours.

Tissue blocks were trimmed and 2 micron sections were cut on an LKB paramatome to be stained in 1% toluidine blue for examination by light microscopy. Thin sections of the selected tissue areas were then cut on an LKB-III ultratome in the gold colour region using glass knives. Sections were mounted on coated copper grids and were double stained with uranyl acetate and lead citrate. Stained sections were then examined under a JEOL, JEM-100C or corinth-275 type electron microscope.The various tissue processing and specimen preparation techniques were according to the procedures outlined by Glauert (1975) and Hayat (1978).

3.4. TEST WATER / DILUTION WATER

A synthetic dilution water was used in most of the experiments to avoid any possible fluctuations in the water quality that could be expected by the use of tap water and at the same time

to maintain the uniformity in the water quality conditions (Alabaster and Lloyd,1980)

Standard dilution water of desired hardness was prepared according to the procedure outlined and recommended for the toxicity tests by the Ministry of Housing and Local Government (1969). Accordingly, three stock solutions were prepared dissolving different chemical salts in deionized distilled water of conductivity less than 10 microsiemens and making up the volume to one litre. Stock solution 1 contained 400g $CaCl_2$, 36g NaCl and 11g $NaNO_3$ in one litre. Stock solution 2 contained 189g $MgSO_4$. $7H_2O$ and 99g $NaNO_3$ in one litre. Stock solution 3 contained 34g $NaNO_3$ in one litre.

For a standard water of 25 mgl^{-1} hardness as $CaCO_3$, 10ml each of stock solutions 1 and 2, and 100ml of stock solution 3 were added to 100 liters of deionized water. Similarly for a standard water of 50 mgl^{-1} hardness 20ml each of stock solutions 1 and 2, and 100ml of stock solution 3 were added to 100 liters of deionized water. In experiments where higher hardness water was needed a standard dilution water of 250 mgl^{-1} hardness as $CaCO_3$ was prepared by the addition of 100ml of stock solutions 1 and 2, and 2000ml of stock solution 3.

All the salts used in the preparation of stock solutions were of analytical grade (BDH Analar). A Gallenkamp Ionmiser-2C deionizer with expendible mixed bed deionizing resin cartridge was used to deionize tap water. In all test water preparations deionized water of conductivity less than 10 micromohs were used by replacing the used resin in the cartridge with fresh

resin as and when the conductivity exceeded 10 μs cm^{-1} (microsiemens).

The chemical composition of the dilution water of 50 ppm hardness as analysed by the Forth River Purification Board and reported by Hasan (1986) given in Table. 3.1.

3.5. TOXICANT

Ammonium chloride was used as the source of ammonia in all the experiments. Large numbers of investigators have used ammonium chloride as the source of ammonia in ammonia toxicity studies. Thurston *et al.* (1983) used three different ammonium salts in their acute ammonia toxicity study and found no significant difference in the toxicity caused by these three different compounds. The chemicals they used were ammonium bicarbonate (NH_4HCO_3), ammonium hydrogen phosphate ($(NH_4)_2HPO_4$) and ammonium sulphate, $(NH_4)_2SO_4$. All through the present experiments ammonium chloride was used as the source of ammonia to maintain uniformity.

To obtain the needed experimental unionized ammonia levels at the measured pH and temperature values of the water, required total ammonia levels were calculated from the following relationship.

$$\text{Total ammonia} = \text{UIA } (1 + \text{antilog } PKa - pH).$$

Then the appropriate amounts of ammonium chloride required to give the total ammonia levels expected were calculated. In calculating the available total ammonia nitrogen per unit weight

of ammonium chloride (NH_4 Cl) the value 1mg NH_4 Cl = 0.2618mg total ammonia-nitrogen was used (APHA 1985). Analytical grade anhydrous ammonium chloride (BDH chemicals) dried at 100°C for 12 hours was used to obtain near exact values of total ammonia to those of calculated values.

3.6. SOURCE OF FISH AND MAINTENANCE

3.6.1.COMMON CARP

Common carp, Cyprinus carpio, L., obtained from a number of hatcheries from the south of England, were used in all the present experimental investigations. Fish on arrival were checked for parasites and for the presence of any possible bacterial or viral infectious agents. Fish were given a prophylactic treatment of either formalin and or malachite green, only in the presence of any ectoparasites or external fungus infections. The fish were kept in quarantine for a period of 15 days before they were transferred to the tropical aquarium. Fish were then held in an independent water-recirculatory fish holding system housed in the tropical aquarium of the Institute of Aquaculture and maintained at water temperatures of between 25-27°C. Fish were held in the aquarium for at least a few months before they were used in the experiments. During this holding period the fish were fed adlibitum twice a day with a commercial trout pellet (EWOS Bakers Omega, no.3; Protein content 47%). Periodically the fish were checked for parasites and any other infections.

3.6.2. RAINBOW TROUT

Young rainbow trout, *Salmo gairdneri* R. used throughout the present experiments were obtained from different commercial trout hatcheries in Scotland as and when required. Fish from a single stock were used at any one time in a particular experiment to maintain a uniform population in all the treatments.

Initial attempts to obtain the fish stock well in advance of experimentation and their maintenance in a water flow through system utilizing public tap water resulted in the development of severe lamellar fusions and hyperplasia and hypertrophy of the gill tissues. Therefore this idea was abandoned and for all experimental purposes the fish were obtained only one week prior to the experimentation and were directly transferred in to acclimation tanks containing synthetic dilution water maintained at 15°C. Each of these tanks were provided with an Eheim filter pump filled with filter material, the water in the acclimation tanks being partially changed every day with freshly prepared dilution water.

3.7. ANAESTHESIA

All experimental fish were anaesthetized before they were sacrificed for histological and electron microscopical studies. Other killing techniques such as concussion and decapitation were totally avoided. Anaesthesia was accomplished using benzocaine (methyl para-amino benzoate). A concentration of 250 ppm was used for carp weighing about 20g. At this concentration 15-25 seconds were required for the immobilization of fish and

a further 15-20 seconds were required for the immobilization of fish and a further 15-20 seconds for the cessation of opercular movements, before the fish were handled.

However for trout, weighing about 10-15g a concentration of only 150 ppm benzocaine was sufficient to produce the same effects.

3.8. TERMINOLOGY

Since the terminology used to represent different forms of ammonia, with associated abbreviations, and the terminology associated with the descriptions of gill tissues are quite variable and often confusing, throughout the text of this thesis the following terminology has been adopted after Emerson *et al.* (1975) and Hoar and Randall (1984) for ammonia and gills respectively.

TERMS USED IN DESCRIBING AMMONIA

NH_3 will be referred to "unionized ammonia", (UIA).

NH_4^+ will be referred to "ionized ammonia" or "ammonium ion".

The sum of (NH_3 + NH_4^+) will be referred to as "total ammonia" (TA) or simply ammonia.

The concentrations of total and unionised ammonia are quantitatively represented as mgl^{-1}, being expressed on nitrogen basis.

TERMS USED TO DESCRIBE DIFFERENT GILL COMPONENTS

"Lamella" (singular): refers to secondary lamellae.

"Lamellae" (plural): refers to secondary lamellae.

"Filament" = refers to primary lamella.

Proximal lamellae = lamellae proximal to arch.

Distal lamellae = lamellae distal to arch.

Interlamellar space = space between lamellae.

Inter filamental space = space between two filaments.

Filament epithelium = primary epithelium or the epithelium that covers the filament including inter lamellar space, in which case it may also be referred to as interlamellar epithelium.

Lamellar epithelium = secondary epithelium, the epithelium that covers the secondary lamellae, also referred to as respiratory epithelium.

Leading edge = filament efferent side.

Trailing edge (refers to water flow direction) = filament afferent side).

Table.3.1. Chemical composition of the 50 ppm dilution water used in carp ammonia toxicity tests.

Parameter	Mean	Range
Total hardness as mgl^{-1} $CaCO_3$	58.50	57.0-60.0
Total alkalinity, mgl^{-1}	42.5	40.0-45.0
as $meql^{-1}$	0.85	0.8-1.2
Conductivity $\mu S\ cm^{-1}$	219.5	194-245
Chloride	31.5	31.0-32.0
Orthophosphate	<0.01	<0.01-0.01
Calcium	7.05	6.9-7.2
Magnesium	3.65	3.2-4.1
Potassium	0.55	0.5-0.6
Sodium	28.3	____
Copper	0.01	0.01-0.01
Iron	0.02	0.02-0.02
Lead	<0.005	
Manganese	<0.005	
Zinc	0.009	0.005-0.017

*All values are in mgl^{-1} unless otherwise stated.

CHAPTER 4

EFFECTS OF AMMONIA ON THE GILL STRUCTURE OF CARP (*CYPRINUS CARPIO*)

4.1. INTRODUCTION

The importance of the deleterious effects of ammonia on carps in general and common carp in particular have received little attention. Common carp is one of the most extensively cultivated fish world wide (Jhingran, 1985; Jhingran and Pullin, 1986) and its importance as a food fish and a potential aquaculture species is growing due to an ever increasing number of hatcheries and carp growing facilities. Hence there is a greater need for an understanding of the effects of most common pollutants such as ammonia on this species.

Although there are a few studies available on the toxicity of ammonia to carp (Vamos, 1963; Daneker, 1964; Flis, 1968; Rao, Rao and Prasad, 1975), according to Dabrowska and Sikora (1986), the studies performed on carp so far are fragmentary. This comment particularly applies to histological studies in which concurrent parasitic infections have also been present (Kovács-Gayer, 1984; Hornich and Tomanek, 1983) and simple dose effects have been performed without any details of corresponding cellular changes (Rao *et al.*, 1975; Dabrowska and Sikora,1986; Hasan and Macintosh, 1986). Furthermore there is a considerable inconsistency and variation within the reported toxic ammonia levels.

Table 4.1. gives the reported toxic effect levels of ammonia to common carp by various investigators, and it is clear from the table that the reported toxicity values vary considerably and more recent works have placed the LC50 values at an appreciably higher level than earlier reporters.

Table 4.1 A summary of the reported toxic effect values of Ammonia to Common Carp

Author	Fish Size (Length/Weight)	PH	Temperature (^{o}C)	NH_3-N mgl^{-1}	Toxic Effect
Vamos 1963	6-8g	8.2	22-25	0.43-0.55	Toxic: Fish sink to the bottom in 60-75 mins.
Danecker 1964 (Cited by Alabaster and Lloyd 1980)	-	-	16	1.23	Lethal in less than 2 days
Flis 1968a	125-260g	8.50	10.75	0.92-1.25	10 days LC16, LC18
Flis 1968b	125-260g	8.05	11	0.11	35 days LC8
Rao et al., 1975	4-5cms (1-2 grams approximately)	7.2-7.8	27.6	1.21 1.06 0.96	24-h TLm 48-h TLm 96-h TLm (= LC50)
Dabrowska and Sikora 1986	23.6-42.5g	7.6-9.2	12.9-13.7	0.91-1.56*	48-h LC50
Hasan and Macintosh, 1986	0.2-0.3g	7.70	28.0	1.74-1.84	96-h LC50 to 168h

*Recalculated values of unionised ammonia (based on the mean temperature, pH and total ammonia values, and according to the formulae of Emerson et al 1975) as the UIA values calculated by the authors (1.15-1.96 mgl^{-1} UIA) appears to have been incorrectly calculated.

Of the two notable earlier studies on the toxic effects of ammonia on carp tissues (Flis, 1968a,1968b ; Kuhn and Koecke, 1956), Flis (1968a,b) described severe regressive changes in most of the carp tissues and gills in particular, whereas Kuhn and Koecke (1956), although noting similar regressive changes but initially used fish with abnormally altered gill structures in their experiments, describing them as normal gills. No further attention has been given to the study of the effects of ammonia on carp gills under controlled experimental conditions. In the past two decades relatively little work has been done on the toxic effects of ammonia on carp gills. The descriptions of normal gill features of carp are also lacking and only a few groups of fish species such as eels and salmonids have been extensively studied.

In addition to the existing paucity of information on the ammonia-induced gill changes in carp, the confusion generated by the recent studies of Mitchell and Cech (1983) and Daoust and Ferguson (1984) in trout and channel catfish (a respectively, that ammonia does not cause any gill lesions has further emphasised the need for investigations in carp. Hence the objectives of this experiment were to investigate the precise role played by ammonia in the absence of other metabolic toxic agents in causing gill damage, and to describe the cellular pathology involved by both light microscopical and electron microscopical investigations.

4.2. MATERIALS AND METHODS

4.2.1.EXPERIMENTAL ANIMALS

The source of the experimental carp, the procedures of their

quarantine, and their maintenance in a recirculation system in the tropical aquarium of the Institute of Aquaculture are described in chapter 3.

Two experimental trials were conducted independently, using two different stocks of fish belonging to two different size groups. The first initial experiment was aimed at identifying the effects of ammonia on the gill tissues, and the 2nd experiment was conducted to confirm the results observed in the first trial. Carp weighing 18.48 grams (± SD, 5.37), and measuring 10.84 cms (± SD, 1.64) were used in the first trial, whereas the carp used in the second trial weighed 7.65 grams (± SD, 2.13) and measured 8.19 cms (± SD, 0.91).

4.2.2. EXPERIMENTAL SYSTEM

The experimental system consisted of static glass tanks of dimensions 95x35x30 cms, used to hold 85 litres of experimental dilution water. Each experimental tank was provided with a microtonic electronic thermostatic heater (Armitage Bros, Ltd., U.K.) to maintain constant temperature and a large sized air stone to provide sufficient aeration. Each experimental tank was also fixed with an Eheim brand filter pump (John Allen Aquariums Limited, U.K.). The clean Eheim pump used here, without any filter wool, served the purpose of keeping the water in good circulation and well mixed. A 12 hrs light: 12 hrs dark period was maintained throughout the experimental period by an electronically operated lighting control device.

4.2.3. TEST WATER

To maintain constant and uniform water quality in all the experimental tanks and in both trials, synthetic dilution water of hardness 50 mgl^{-1} as $CaCo_3$ prepared by mixing appropriate quantities of stock solutions of chemical salts as outlined in chapter 3 was used. The chemical composition of similar dilution water as analyzed by the Forth River Purification Board, Stirling, is provided in table 3.1 of chapter 3.

To maintain constant pH, the dilution water of all the experimental tanks was buffered with 0.01 M phosphate buffer by addition of appropriate amounts of di-sodium hydrogen orthophosphate and sodium dihydrogen orthophosphate. Similar buffer salts, and dilution water at similar concentrations have been used the ammonia toxicity studies on carp by Hasan and Macintosh (1986) and hence were adopted in the present studies, although a variety of other buffer salts have been used by various workers in ammonia toxicity experiments.

4.2.4. ACCLIMATION AND EXPERIMENTAL PROCEDURE

One week prior to the experimentation,required numbers of carp from the stock held in the tropical aquarium were removed for acclimatisation to the experimental water quality conditions. All the fish were acclimatized to ammonia free unbuffered dilution water of 50 ppm hardness in separate acclimation tanks, of similar type to the experimental glass tanks over a period of 6 days, by partial daily water replacements. During this period the fish were starved and the excreted faecal solids were trapped with the help of an Eheim filter pump,

filled with filter wool to avoid any possible reconsumption of the faecal solids by fish. This process was necessary to maintain the tank waters clean and at the same time to keep the ammonia levels stable. A week of starvation has been shown to stabilize the ammonia excretion rates to a minimum level. (Kaushik 1980; De Vooys 1968). On the seventh day of acclimation process all the fish were also acclimatized to ammonia free buffered dilution water similar to the experimental dilution water for a period of 24 hours (American Public Health Association (APHA) 1980).

4.2.5. AMMONIA CONCENTRATIONS TESTED

In the 1st trial three concentrations of ammonia were tested with one control. The concentrations used were 0.50, 1.0 and 1.5 mgl^{-1} as unionized ammonia (UIA). Since there were no mortalities at the highest concentration of ammonia tested in the first trial (ie 1.5 mgl^{-1} UIA), in the second trial, that was intended to verify the histopathological observations noticed in the first trial, a further higher concentration was also tested. The ammonia concentrations tested in the 2nd trial were 0.5, 1.0, 1.5 and 2.0 mgl^{-1} UIA.

4.2.6. REPLICATION

In both the trials conducted each experimental ammonia concentration was tested in duplicate, with a duplicate set of control tanks with no added ammonia.

4.2.7.NUMBER OF FISH AND THEIR DISTRIBUTION

Ten fish were tested in each of the duplicate ammonia test concentrations, and in controls, thus making up a sample size of 20 fish tested at each test concentration. Before the start of the experiment the fish were gently netted out from the acclimation tanks with minimum disturbance using soft meshed shallow nets, and were randomly distributed among the test tanks as recommended by Sprague (1973).

4.2.8. EXPERIMENTAL DURATION AND SAMPLING INTERVALS

Each trial lasted for a period of a full seven days (or 168 hours), fish samples for histopathology being taken at the end of three full days and seven full days of exposure. Five fish were sampled from each of the test tanks on day 4 and day 8.

4.2.9.BEHAVIOURAL OBSERVATIONS

Fish in different test solutions were observed for behavioural changes throughout the experimental period.

4.2.10. MEASUREMENT AND ANALYSIS OF WATER QUALITY

During the experimental period water quality parameters such as pH, temperature and conductivity were measured every day, whereas total ammonia nitrogen and nitrite nitrogen were measured on alternate days. Total alkalinity and dissolved oxygen were measured at the beginning and before the end of the experiments. Total hardness was only measured at the beginning, at the time of reconstitution of dilution water.

The methods employed in the measurement of various physicochemical parameters of the experimental water samples are outlined in chapter 3. Unionized ammonia concentrations were calculated from the measured mean total ammonia concentrations, measured mean temperatures and pH values for individual tanks according to procedures of Emerson *et al.* (1975).

Water samples from each experimental tank were analyzed separately for all the parameters mentioned. From the mean values of the physico-chemical parameters calculated for each individual tank, mean and standard error was computed for each duplicate set of treatment and are tabulated.

4.2.11. TISSUE SAMPLING FOR HISTOLOGY AND ELECTRON MICROSCOPY

Five fish each were sampled for histopathological examination from each experimental tank on day 4 and day 8. Thus at any one sampling period a total of 10 fish were sampled at each test concentration tested in duplicate. Fish were individually netted out with minimum disturbance to the other fish, into an anaesthetic container held close to the experimental tank to avoid the fish being exposed in air and thereby to reduce the possible physical trauma that may lead to any alteration in the gills such as telangiectasis. The anaesthetic used, its concentration and time required for anaesthesia have been detailed in chapter 3. Immediately after anaesthetization the fish were quickly weighed and measured, and the gill samples were taken for both light microscopical and electron microscopical investigations, separately from individual experimental tanks. All the 1st and 2nd gill arches from five

individual fish of a single test tank were pooled together for combined scanning and transmission electron microscopical observations. The rest of the gills from the individual fish from individual tanks were fixed separately for light microscopic histological examination. Details of sampling, fixation and processing for both light microscopic wax embedding and electronmicroscopy are as detailed in chapter 3.

4.2.12. NUMBER OF GILLS EXAMINED

For scanning electron microscopy a minimum of four whole or partially trimmed gill holobranchs from each of the pooled gill samples belonging to each tank were used. Whereas, for transmission electron microscopy although a number of samples were used, only selected area of the required gill regions were sectioned from different ammonia exposed fish.

For light microscopical histological preparations all the gills from each fish were fixed and processed separately and examined in both the trials.
A qualitative assessment of the changes noted are presented.

4.3. RESULTS

4.3.1 WATER QUALITY

Total ammonia and unionized ammonia concentrations in all the treatment tanks remained close to the nominal values (*i.e.* 0.5, 1.0, 1.5 and 2.0 mgl^{-1} unionized ammonia or 17.21, 34.41, 51.62 and 69.45 mgl^{-1} total ammonia as applicable, appropriate to the concentrations in both Trial 1 and Trial 2, throughout the experimental period (Table 4. 2 and Table 4.3).

The ammonia concentrations recorded from the control tanks were those resulting from the fish's own metabolic activity, and the values recorded (0.034 and 0.016 mgl^{-1}, UIA respectively for trial 1 and trial 2) are within the levels that have been reported as nondetrimental to the optimum growth and food conversion efficiency of common carp (Tóth, Gulyás and Oláh, 1982).

4.3.2 MORTALITIES

No mortalities occurred in any of the experimental tanks except at the highest levels of unionized ammonia tested. At the highest level of unionized ammonia tested of trial 2, all of the fish from the replicate tanks died within 24 hours, except one fish that survived up to 36 hours of exposure.

Table 4.2 Physico-chemical characteristics of the test solutions measured during the carp experimental period (Mean ± SE, n=2 (trial 1)*1

	NH_4Cl mgl^{-1}	Total Ammonia-N mgl^{-1} Mean ± SE	NH_3-N*2 mgl^{-1} Mean ± SE	Temperature ^oC Mean ± SE	pH Mean ± SE	Dissolved oxygen mgl^{-1} Mean ± SE	Total Alkalinity $meql^{-1}$ Mean ± SE	Electrolyte Conductivity $nS\ cm^{-1}$ Mean ± SE	Nitrite-N mgl^{-1} Mean ± SE	Nitrate-N mgl^{-1} Mean ± SE
Control										
	0.00	1.195 ± 0.52	0.034	26.84 ± 0.51	7.66 ± 0.01	7.8 ± 0.21	9.03 ± 0.14	1557 ± 24.75	0.003 ± 0.001	0.697 ± 0.06
1	67.76	18.055 ± 0.26	0.527	27.76 ± 0.06	7.64 ± 0.049	7.88 ± 0.04	9.30 ± 0.34	1895 ± 49.50	0.005 ± 0.001	0.654 ± 0.14
2	131.41	35.76 ± 0.35	1.014	27.34 ± 0.62	7.64 ± 0.01	7.6 ± 0.07	9.28 ± 0.33	1987 ± 5.66	0.006 ± 0.002	0.737 ± 0.125
3	197.18	53.01 ± 0.21	1.526	27.23 ± 0.95	7.65 ± 0.02	7.5 ± 0.07	9.37 ± 0.25	2089 ± 43.84	0.004 ± 0.002	0.654 ± 0.14

*1 The hardness of the dilution water used in these tests remained within 56.0 (SD, 1.65) mgl^{-1} as $CaCO_3$.

*2 Unionized ammonia (NH_3-N) concentrations were calculated from the measured mean values of total ammonia, pH and temperature

Table 4.3 Physico-chemical characteristics of the test solutions measured during the carp experimental period (Mean ± SE, n=2) (trial 2)*1

	NH_4Cl mgl^{-1}	Total Ammonia-N mgl^{-1} Mean ± SE	NH_3-N*2 mgl^{-1} Mean ± SE	Temperature ^oC Mean ± SE	pH Mean ± SE	Dissolved oxygen mgl^{-1} Mean ± SE	Total Alkalinity $meql^{-1}$ Mean ± SE	Electrolyte Conductivity $nS\ cm^{-1}$ Mean ± SE	Nitrite-N mgl^{-1} Mean ± SE
Control									
	0.00	0.66 ± 0.03	0.017	26.54 ± 0.49	7.62 ± 0.007	7.28 ± 0.35	8.91 ± 0.16	1792 ± 38.0	0.007 ± 0.002
1	65.72	18.07 ± 0.04	0.506 ±	27.14 ± 0.09	7.64 ± 0.021	8.45 ± 0.21	9.69 ± 0.10	1925 ± 61.0	0.006 ± 0.0001
2	131.41	34.90 ± 0.05	0.947 ±	27.02 ± 0.02	7.63 ± 0.007	8.5 ± 0.14	9.16 ± 0.34	2133 ± 26.0	0.006 ± 0.001
3	209.5	52.53 ± 1.85	1.528 ±	27.05 ± 0.07	7.66 ± 0.007	8.45 ± 0.64	8.88 ± 1.17	2242 ± 38.0	0.006 ± 0.0002
4	279.25	69.45 ± 0.04	1.990 ±	26.50 ± 0.43	7.67 ± 0.007	7.75 ± 0.49	9.35 ± 0.19	2392 ± 38.0	0.004 ± 0.0001

*1 The hardness of the dilution water used in these tests remained within 53.33 (SD, 2.69) mgl^{-1} as $CaCO_3$.

*2 Unionized ammonia concentrations were calculated from the measured mean values of total ammonia pH and temperature.

4.3.3 BEHAVIOURAL OBSERVATIONS

The control fish remained active and apparently normal throughout the experimental period. During most of the experimental period the control fish remained in groups as they moved around within the tanks, usually at the bottom.

At 0.5 mgl^{-1} UIA

Fish at 0.5mgl^{-1} UIA concentration did not exhibit any abnormal behavioural patterns, they remained less active and in groups with most of them on the bottom of the tank.

At 1.0 mgl^{-1} UIA

Although during the initial few hours, the fish stayed at the bottom, with minor initial distressed movements, they slowly and eventually became distributed all over the tank at various depths of water. After 12 hours most of the fish stayed above the midwater level in the tank, and most often at the surface. Occasional erratic bursts of swimming were noticed. Most fish developed mild nervous signs demonstrated by their unbalanced swimming by tilting over to one side. Occasionally some fish showed fast spiral movements. Although some fish remained like this until the end of the experimental period, a few regained their normal balance between day 5 and day 6.

At 1.5mgl^{-1} UIA

Severe distressed reactions were noticed at this concentration although no mortalities occurred. Most of the fish remained

above the midwater level, and dispersed all over the water column. Erratic movement and sporadic hyperexcitability was characteristic. Most fish developed mild to severe signs of nervous dysfunction with their failure to maintain the normal body position in water. Although increased opercular movement and breathing was noticed during the first 24 hours of exposure, subsequently opercular movements and breathing seemed to slow down considerably. However, periodic coughing associated with short quick bursts of movement was commonly seen. Some fish exhibited great difficulty in swimming, and tended to sink to the bottom,in spite of their efforts to move and hold the body in a normal position. Occasional spiral movements were also noticed.

AT 2.0 mgl^{-1} UIA

Fish reacted vigorously at this concentration and developed nervous signs faster than at 1.5 mgl^{-1} UIA. Before death, fish slowly sank to the bottom of the tank remaining alive for various periods of time before their opercular movements.The first death occurred as soon as 6 hours after exposure, and all the fish except one were dead by about 24 hours. Distressed erratic movements were common throughout. Since most fish had died overnight none were sampled for histology except one moribund on the bottom of tank after 36 hours of exposure. This fish was killed without any anaesthesia and the gills from this fish were processed for histology and scanning electron microscopy. No dead fish were sampled at any time.

4.3.4. HISTOPATHOLOGY

The results of the present experiments as visualized by means

of combined histological, scanning and transmission electron microscopical investigations were of particular interest.

The observations noted for each experimental treatment were distinctive. The control fish showed changes that were not present in the ammonia treated fish (but for the very minimal extent detailed in the later part of this text) or in any of the unexposed stock fish, whereas the lesions characteristic of ammonia exposure increased from fish exposed to 1.0 mgl^{-1} UIA to 1.5mgl^{-1} UIA, but were absent at 0.5mgl^{-1} UIA (Plate, 1).

In the current experiment, as a result of the undesired effects of the buffer on the gill epithelium of carp, true experimental control gills could not be obtained. However for the purpose of comparison, some normal gill structural features were studied from a sample of stock fish (same stock carp as used in this experimental trial-1) by scanning electron microscopy are given in appendix 1. In the present investigations it was also noted that the normal morphological features of trout gills varied slightly from those of carp. Normal trout gill structure as noted by an SEM study using healthy rainbow trout of 40-45 gram size included in appendix 1.

Fish exposed to 1.0 and 1.5 mgl^{-1} UIA developed mild to severe histological changes in the gill tissue. The major pathological changes were the appearance of hyperplastic and hypertrophic swellings on the gill raker, gill arch and gill filament epithelium. These swellings remained small and few in number in the case of fish exposed to 1.0 mgl^{-1} UIA (Plate 2), whereas in fish exposed to 1.5 mgl^{-1} UIA, the swellings

appeared as large and unevenly raised areas of the epithelium all along the length of the filament (Plate 3). These lesions were present all over the afferent and efferent sides of the filaments on raker epithelium, but were fewer in numbers on the arch epithelium. However they were not found on the lamellar epithelium. Scanning electron microscopy revealed that the epithelial cells on these raised areas had either totally or partially lost their microridges and the epithelium in some cases was perforated (Plate 4). The lesions as observed in thin sections consisted of variably sized local enclosures and contained different breakdown stages of epithelial, chloride, mucous, eosinophilic granular and infiltrated mononuclear cells (Plate 5a). The progression of these lesions resulted in the formation of a syncytium comprised of cellular debris in a heterogeneous protoplasmic matrix and often contained aggregations of intact nuclei (Plate 5b). The lesions on the filament epithelium were complex in that there was an involvement of various types of cells as detailed above, whereas on the gill rakers they consisted of simple large epithelial swellings filled with a sparsely dense, homogeneous, and slightly PAS positive matrix containing mucous and epithelial cell debris(Plate 6). Large numbers of these swellings were noticed on the gill arches by day three but were decreased by day seven, whereas the swellings on the filament epithelium increased in density from day three to day seven. Large numbers of sloughed intact nodules and necrotic cellular debris could be seen in the inter-filamental areas in histological sections (Plate 3c,d.).

Chloride cell proliferation and necrosis was another major pathological change seen in the gills exposed to higher ammonia concentrations. Although gills exposed to 0.5 mgl^{-1} UIA showed very mild chloride cell swelling and proliferation on the secondary lamellae, in fish exposed to 1.0 and 1.5 mgl^{-1} UIA large scale proliferation of chloride cells both on the lamellae and filament epithelium was seen (Plates 7, 8 and 9). At the highest concentration of ammonia chloride cells often formed a continuous array on the filament epithelium with interconnections between the adjacent and neighbouring chloride cells. Ultrastructural investigations revealed two types of chloride cells; electron lucent pale staining cells and electron dense dark staining cells(Plate 10a). The pale cells showed degenerative changes such as pyknosis of the nuclei, mitochondrial swelling and disappearance of mitochondrial cristae, causing the mitochondria to look like homogeneous structures(Plates 10b,11a and 11b). Another characteristic feature of the pale cells was the appearance of dark inclusion bodies and greatly reduced cellular organelles and tubular structures. The dark staining cells were packed with mitochondria and a dense tubular system with minor changes in the cell components (Plate 11).

Plate 1. Scanning electron micrographs of carp gills after 7 days exposure to ammonia concentrations of 0.5 mgl^{-1} as UIA. (Note the normal appearance of the gill filament and lamellar epithelium)

(a) 30 X

(b) 700 X

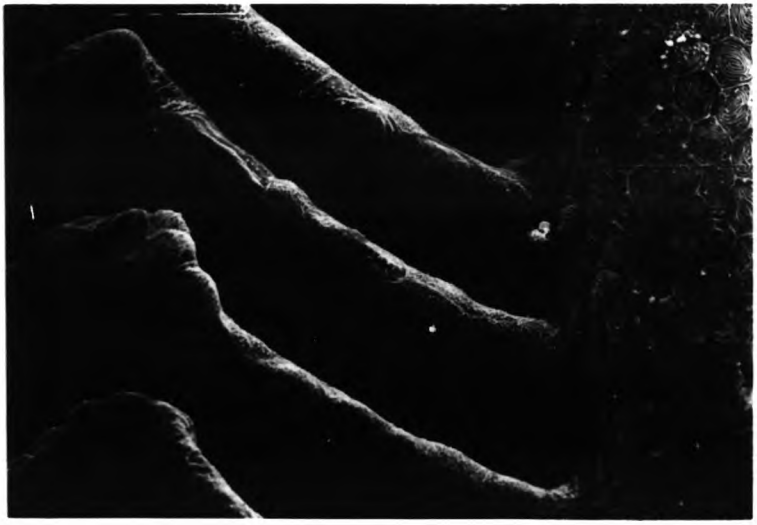


Plate 2. Scanning electron micrographs (SEM) of carp gills after 7 days exposure to a unionized ammonia concentration of 1.0 mgl^{-1} UIA

a) Note the appearance of small nodular swellings on the filament epithelium (arrowed) 600 X

(b) Nodular swelling with obvious damage to the epithelial cells 6,000 X


b

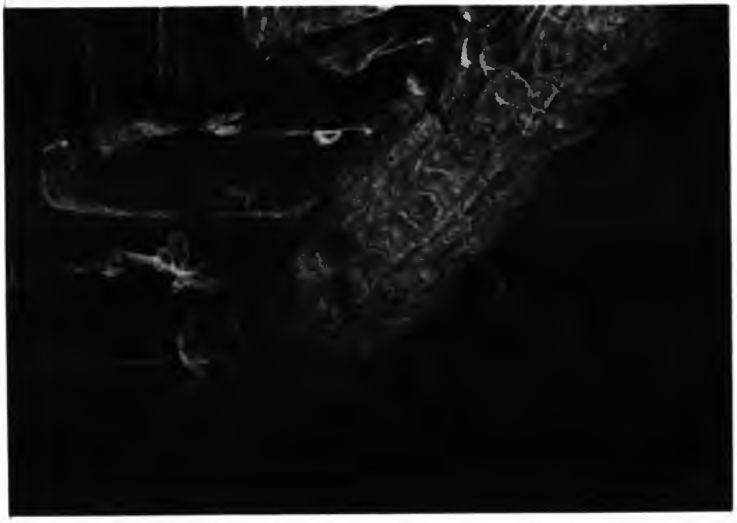

a

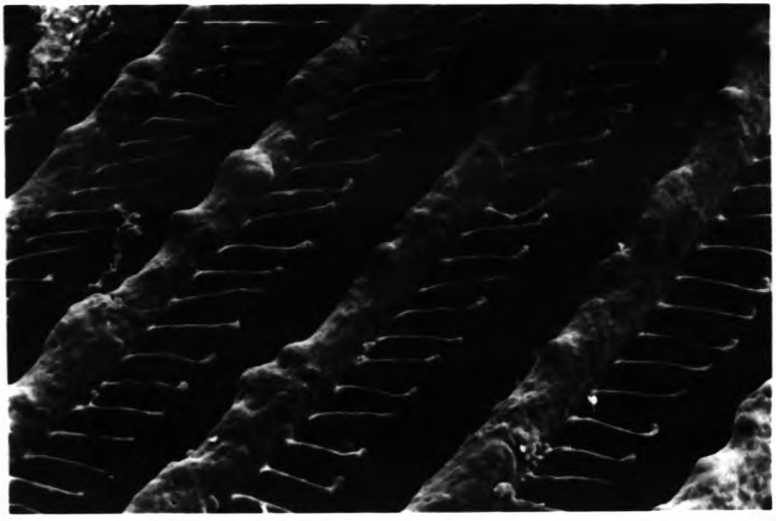
Plate 3. Nodular lesions on the gill epithelia of carp exposed to 1.5 mgl^{-1} UIA, for 7 days.

(a) Extensive and proliferative nodular swellings extending along the length of filament epithelium seen after seven days exposure.(SEM, 60 X)

(b) At high magnification, the swellings are seen as both hyperplastic and hypertrophic nodules on the filament epithelium with apparently normal looking lamellae (SEM, 240X).

(c) Photomicrograph of a histological section showing the nature of the extensive nodular lesions on the gill filaments (H&E, 150X).

(d) Photomicrograph showing the necrotic cells and a sloughing nodular swelling (H&E, 600X).



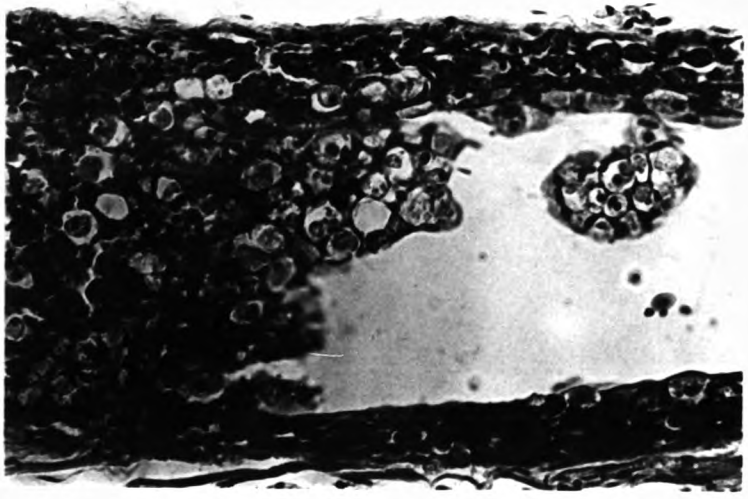


Plate 4. SEM of gill nodular lesions from carp exposed to 1.5 mgl^{-1} UIA
for seven days.

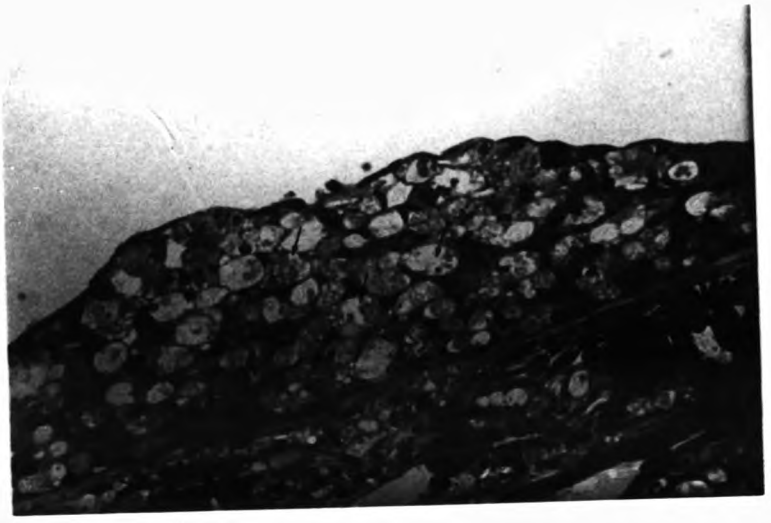
(a) Nodule on the filament epithelium showing erosion of microridges 2300 X

b) Nodule on gill arch epithelium (note the erosion of microridges and perforations of the squamous epithelial cells) 2300 X

Plate 5. Photomicrographs of the developing nodular lesion on the gill filament epithelium of carp exposed to 1.5 mgl^{-1} UIA,

(a)Initial infiltration of EGCs (arrowed) and other cell types resulting in swelling of the epithelium. (Toluidine Blue, 600 X)

(b) A progressive stage in the development of the lesion resulting from the necrosis and dissolution of the cell membranes leading to a homogenous mass of structure representing a "syncytium".
Note the intact nuclei (arrowed) within the protoplasmic matrix (Toluidine Blue, 1500 X).



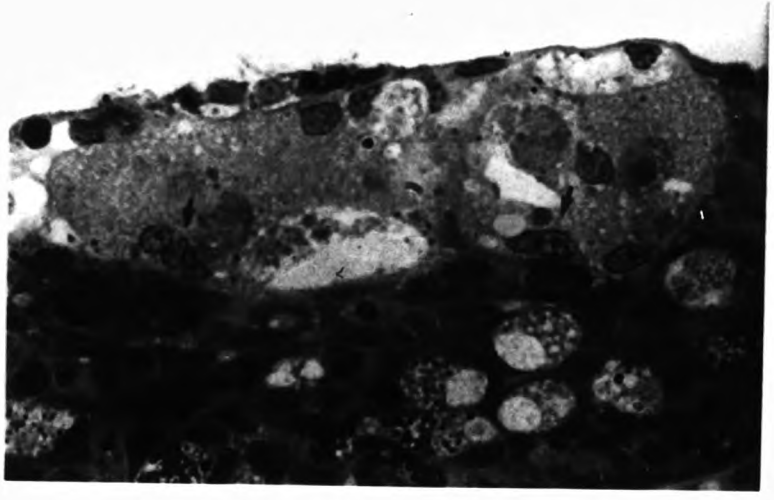
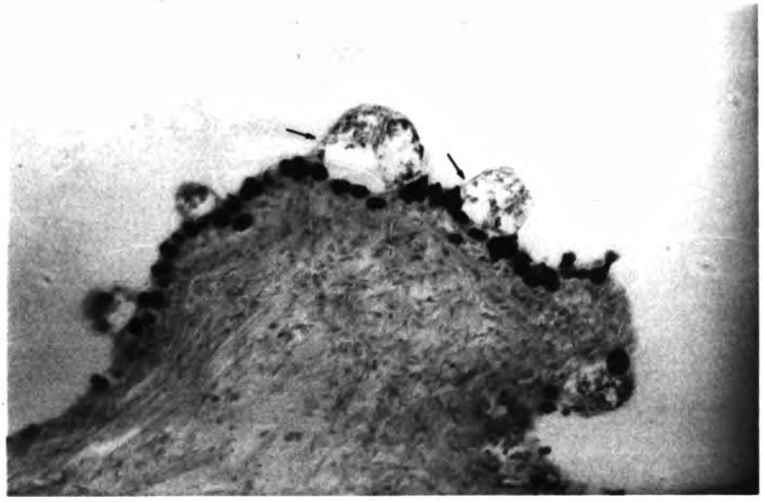


Plate 6.Photomicrograph of the nodular lesions on the gill aker epithelium of carp exposed to 1.5 mgl^{-1} UIA for 3 days (arrowed)

(a) Nodule on the tip of the gill rakers. Note the involvement of fewer cells in the lesion formation and the PAS positive matrix (PAS, 375X)

(b) Similar lesions on the gill rakers (PAS, 600 X)



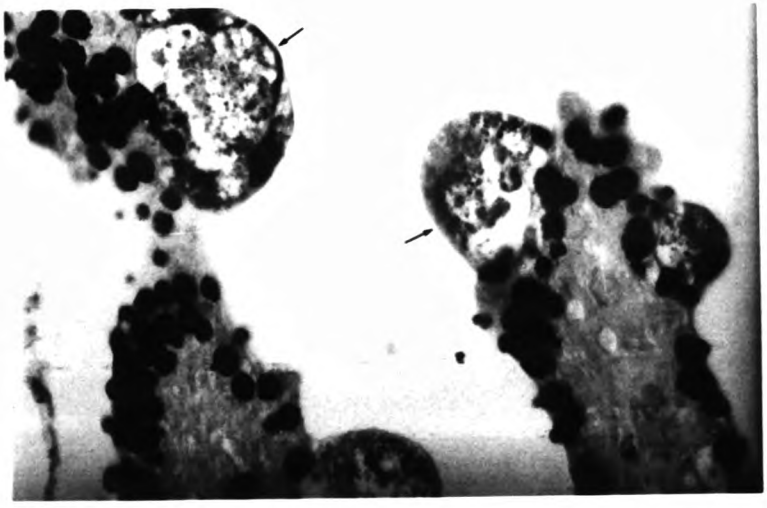
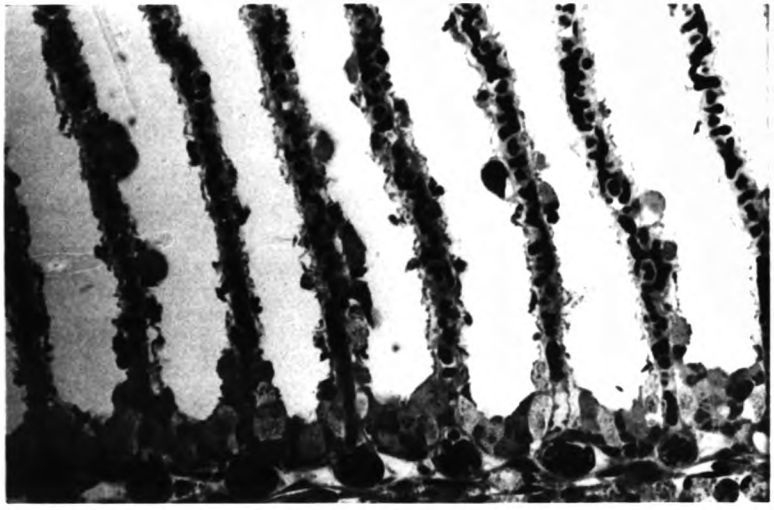


Plate 7.

(a) A photomicrograph of the gill lamellae of carp exposed to 1.5 mgl^{-1} UIA for 7 days showing severe chloride cell proliferation on lamellae with necrotic cells, (Toluidine Blue 675 X)

(b) Photomicrograph of the gill filament of carp exposed to 1.0 mgl^{-1} UIA for 7 days showing moderate chloride cell proliferation on the lamellae (arrowed), (H&E, 375 X).



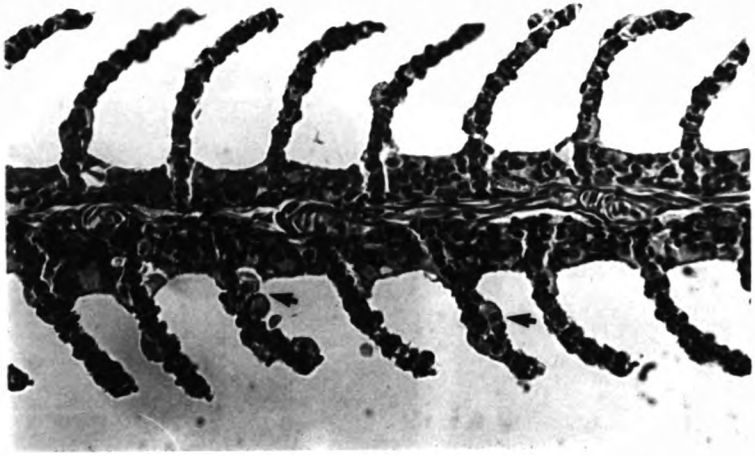
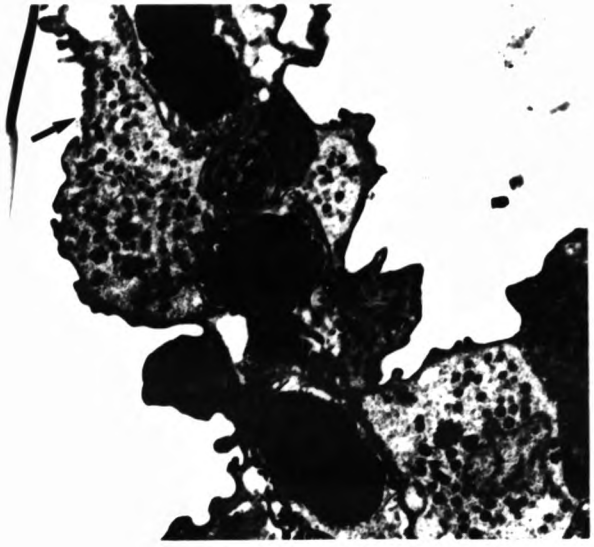


Plate 8. Transmission electron micrographs of carp gills exposed to 1.5 mgl^{-1} UIA showing chloride cell proliferation

(a) on the lamellae and

(b) in the interlamellar regions.

Note the abundance of mitochondria (M) and apical crypts opening to the exterior (arrowed) 4750 X



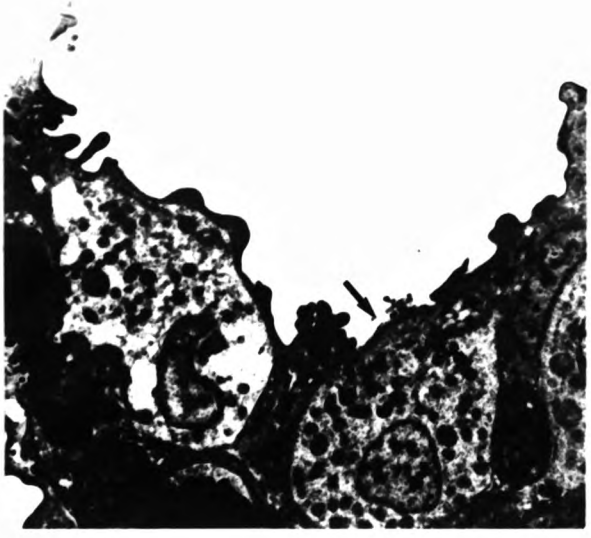


Plate 9.

(a) A photomicrograph of the chloride cell proliferation in the interlamellar and filament epithelial region of carp exposed to 1.5 mgl^{-1} UIA for seven days (PAS, 1.500 X).

(b) A TEM carp gill filament epithelium showing the development of a continuous array of chloride cells with interconnecting channels after seven days exposure to 1.5mgl^{-1} UIA. Note the conspicuous dark inclusion bodies (2850 X).

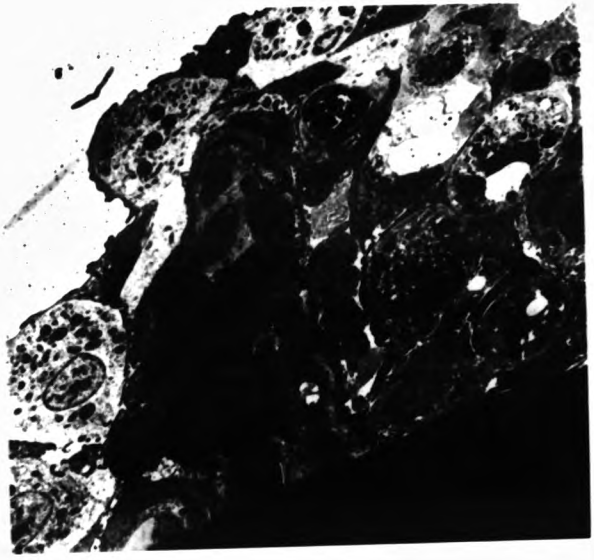
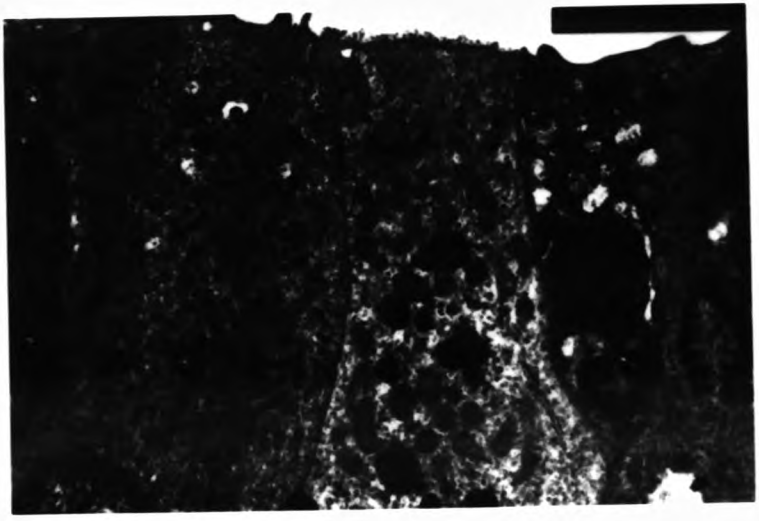


Plate 10. Transmission electronmicrographs of gill filament chloride cells from carp exposed to 1.5 mgl^{-1} UIA for seven days

(a) TEM showing electron lucent (P) and electron dense (D) chloride cells. Note the dilated mitochondria in the pale staining (electron lucent) cell with numerous dark inclusions and thinning of the cytoplasmic organelles and dilation of the tubules, where as in the dark staining cell such changes are minimal and contain numerous mitochondria (5010X)

(b) Chloride cells showing pyknotic nuclei and marked dilation of the micro tubules (4050 X)



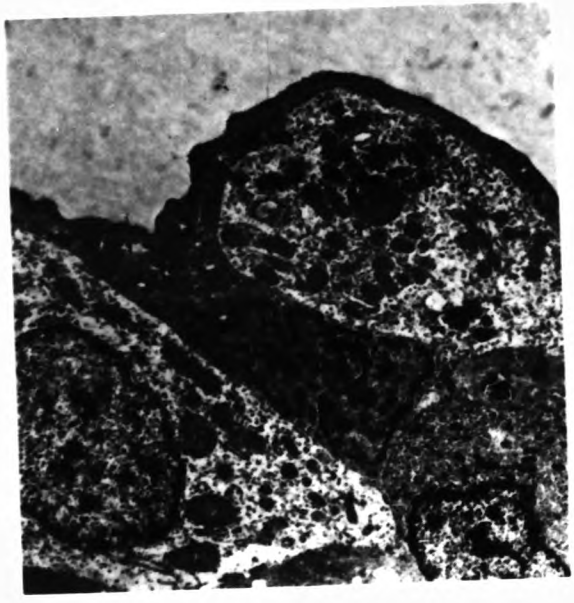


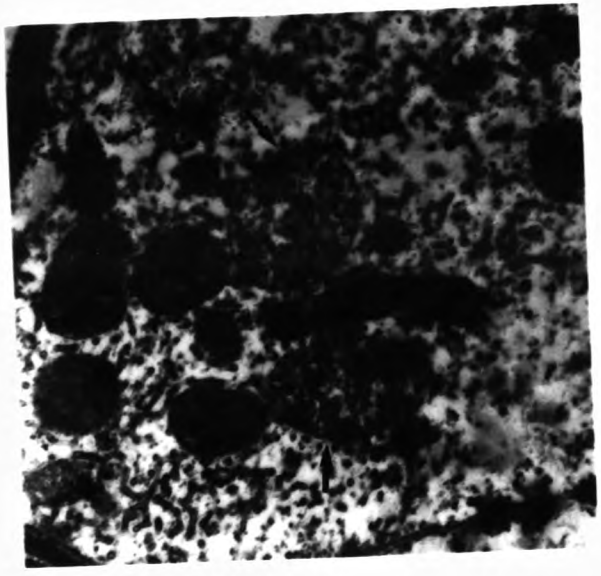
Plate 11.

TEM of chloride cells from the carp gills exposed to 1.5 mgl^{-1} UIA for 7 days

(a) Note the pyknotic and karyolytic nuclei (N) with both normal mitochondria and necrotic mitochondria with no mitochondrial cristae (arrowed) 6000 X

(b) Mitochondrial necrosis characterized by the dilated and swollen mitochondria (arrowed) 6150 X


N.

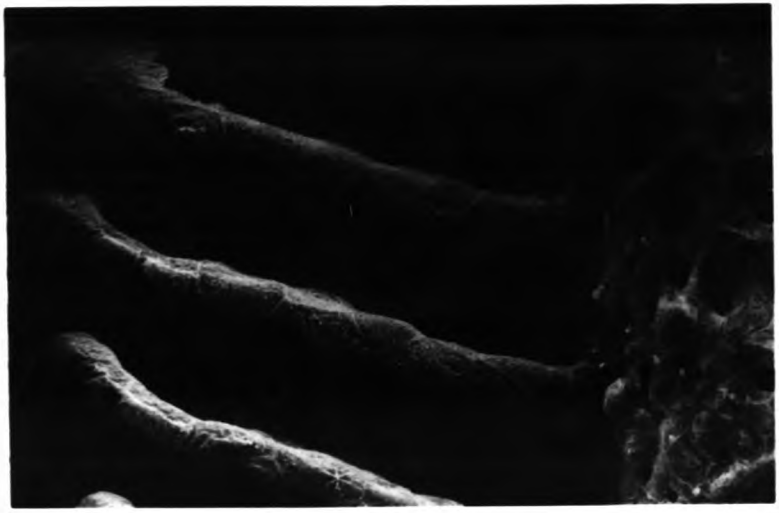


Mucous cell proliferation and an increased mucous cell activity was also noticed. There was an increase in the mucous cell activity with an increase in the unionised ammonia. At 1.5 mgl^{-1} UIA the mucous cells appeared more exhausted. The openings of such mucous cells were flared (Plate 14). At 1.0 mgl^{-1} UIA the mucous cells were active but the mucus appeared to plug the opening of the cell (Plate 13). Although there were large numbers of mucous cell openings at 0.5 mgl^{-1} UIA they were normal and devoid of any mucus plugs under SEM, indicating that the mucus was of normal viscosity and would have been washed off or lost in the course of processing for SEM (Plate 12).

The other important change was that of an intensive infiltration of eosinophilic granular cells into the gill epithelium and connective tissue. They were also present along the central axis of the gill filament. Degranulation, degeneration, and severe vacuolation processes at different stages were present (Plate, 15). Once again their intensity and numbers increased with increasing levels of UIA. In the connective tissues of arch, gill septum and along the filament axis, large numbers of mononuclear cell infiltrations were seen in addition to eosinophilic granular cells.

Plate 12.

SEM of carp gill filament and arch epithelium showing normal mucous cell openings and normal epithelial cell pattern after 7 days exposure to 0.5 mgl^{-1} UIA. 1200 X



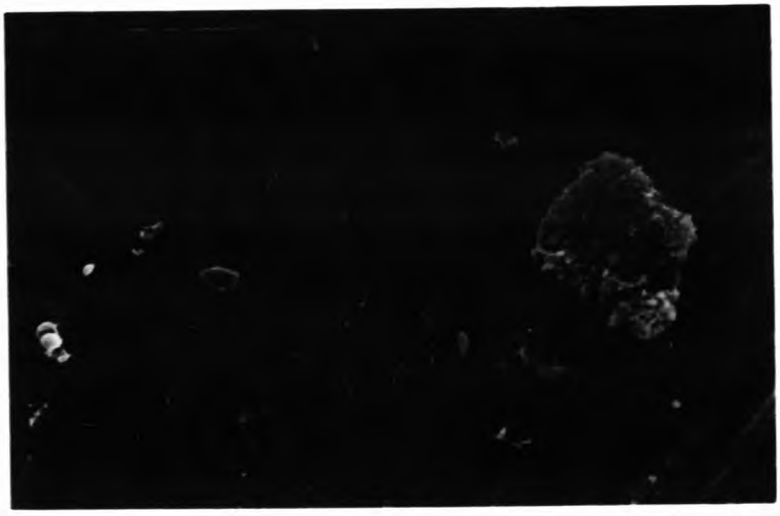


Plate 13.

SEM of carp gills exposed to 1.0 mgl^{-1} UIA for 7 days. Note the thickening of the mucus that has resulting in the blocking of the mucous cell openings.

(a) Gill arch epithelium 1900 X

(b) Gill filament epithelium 400 X

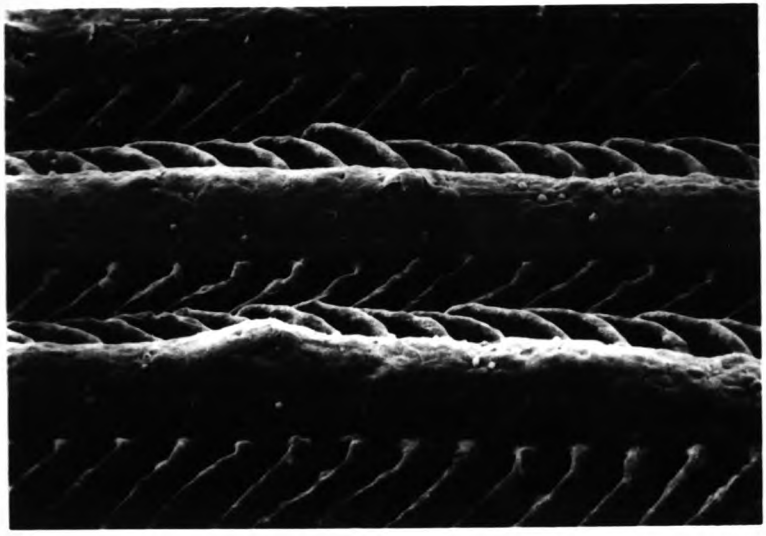


Plate 14. SEM of carp gill epithelial surface showing severe exhaustion of the mucous cells at 1.5 mgl^{-1} UIA after 7 days exposure

(a) Gill filament epithelium 1800 X

(b) Gill arch epithelium showing both exhausted mucous cell openings and those with thick mucus plugs 600 X

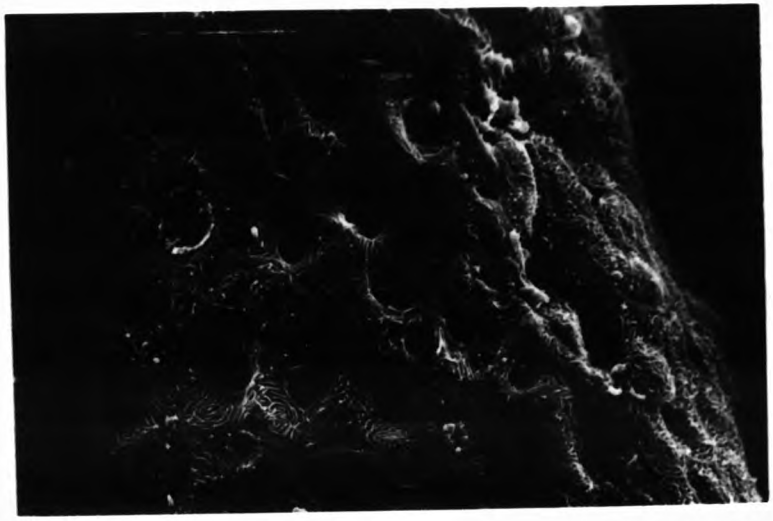
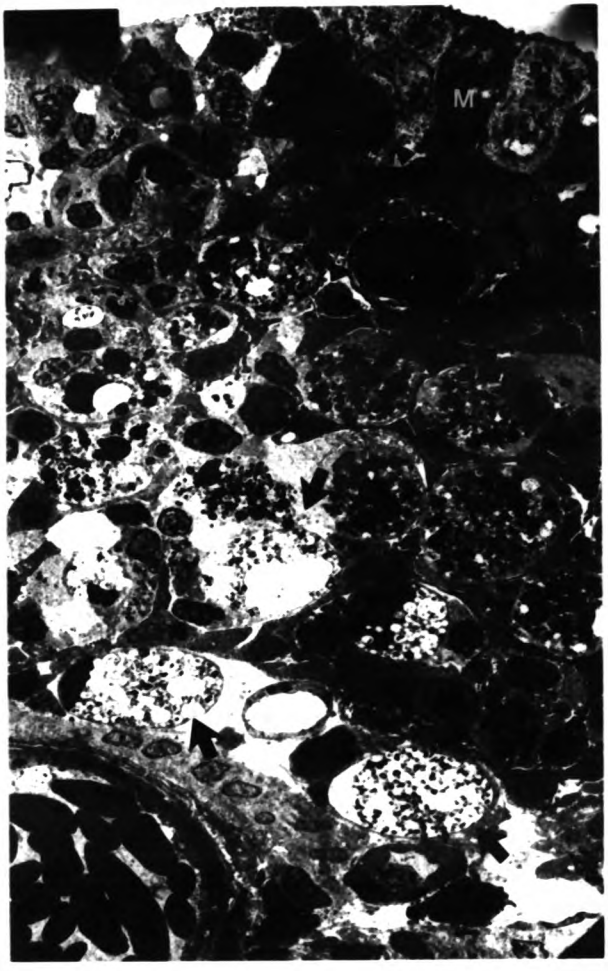


Plate 15.TEM of transverse section of the gill arch from carp exposed to 1.5 mgl^{-1} UIA for seven days showing severe infiltration of the eosinophilic granular cells. Note the EGCs in various stages of degranulation (arrowed). Also could be seen are the darkened condensed cells (arrow heads), necrotic chloride cells (CC) and mucous cells (M).


M

The control gills were quite variable in their appearance,but the majority of the lamellae and filaments showed severe alterations both at day three and day seven. These alterations were not in any way comparable to those found in the fish gills exposed to ammonia.

The characteristic change was variable to severe hyperplasia of the lamellar and filament epithelium giving an abnormally thickened appearance to lamellae and filaments. Due to this severe proliferation of the epithelial cells, only very short lengths of the lamellae were free, the rest being buried within the thickened epithelium, particularly towards the tips of the filaments (Plate 16). There was an oedematous expansion of the lamellar thickness with the appearance of wide spaces from the base of the lamellae towards the tip. This was associated with an intense and active migration of fibrocyte like cells into the lamellae from the basal regions of interlamellar epithelium on either side of the pillar capillary basement membrane (Plate 17). These cells seemed to be transforming into a different type of active mucous cell. Apart from these proliferative and oedematous changes, another conspicuous change was an increased infiltration of eosinophilic granular cells into these intercellular areas. Although some of these granule cells remained within the cartilaginous tissue and contained partially opened granules, probably indicative of the release of the contents of the granules, the granule cells within the filament epithelium and those around the basal regions of the lamellae remained intact, containing a conspicuously electron dense central core (Plate 18).

Gills from fish exposed to low ammonia concentrations (0.5 mgl^{-1}) showed only minor alterations, including the presence of a few fibroblast cells in the basal regions of the normal looking lamellae. These fibroblasts neither showed any further development into mucous cells, nor migration further up into the lamellae, but rather appeared to remain stationary. The other most important details were the absence of cellular hyperplasia of the lamellar or filamental epithelia, and excessive infiltration of EGC's into these epithelia as noted in the control fish gills. The density of the EGC's in the epithelia remained moderately low when compared to either the control fish or those exposed to higher levels of ammonia. However, a moderate increase in the chloride cells was seen especially in the interlamellar and basal areas of the lamellae. No lesions characteristic of hyperplasia and hypertrophic swelling as noted in the high ammonia exposed gills were present at this ammonia concentration (Plate 1). The lamellae were least affected and remained normal in appearance. A moderate increase in the number of mucous cells was noted however, on the filament and gill arch epithelia both in day 3 and day 7 samples.

Thus the overall appearance of the gill structure remained more normal at 0.5 mgl^{-1} UIA than either in the control fish or those exposed to 1.0 or 1.5 mgl^{-1} UIA.

A cell type that has been described as the "rodlet cell" and was structurally different from the surrounding tissue cells was observed in the gill tissues of both control and ammonia treated carp. These rodlet cells were pear shaped or oval with a relatively thicker cell border than the surrounding cells,

including those of EGC's. They contained an eccentric or polar spherical nucleus, and a varying number of tapering rod-like structures within the cytoplasm. The basal part of the cell was enlarged and the rods were orientated with their tapering ends towards the apex of the cell.

Although these cells stained well in H&E they were relatively less conspicuous and hence difficult to distinguish in the histological sections. They stained negatively for both PAS and AB stains. A modified methylene blue and basic fuchsin procedure of Dougherty (1981) as used by Vallejo (1987), unsuccessfully to demonstrate EGCs in rainbow trout gave a strong staining to the rodlet cells, and thus it was adopted for the easy demonstration and visualization of rodlet cells in this study. As noted (Vallejo,1987) although this stain does not stain eosinophilic granule cells specifically, some mucous cells and the cartilage, stain an intense dark blue, while the rest of the tissue stains uniformly light blue. However, the characteristic pear-shaped rodlet cells, with their sickle shaped rodlets, eccentric spherical nuclei and a dense staining cell membrane appear relatively more conspicuous and easily distinguishable.

In the carp gills, the rodlets were noted particularly in the gill arch connective tissues, especially in the supporting tissues of blood vessels and septal connective tissues.The number of rodlets in the normal and control carp were fewer (Plate 19a). However, their numbers increased considerably in the gill tissues of the fish exposed to 0.5 mgl^{-1} UIA (Plate 19 b). In the gill tissues at this ammonia concentration the rodlet cells were more frequently seen in the connective

tissues, sometimes appearing in small aggregations. They appeared to be commonly associated with blood vessels, being present in large numbers in the vicinity or sometimes within the endothelium of the blood vessels in single or multiple layers. Further, since there were relatively fewer granular cells in the connective tissues, at this concentration of ammonia the rodlet cells appeared clearer, and they were also very discrete with conspicuous rodlets.

With increasing ammonia concentrations, the rodlets appeared to be disrupted. At 1.0 mgl^{-1} UIA, although a larger population of rodlet cells were clearly visible in the connective tissues, the rodlets within these cells were less discrete in most of the cells, whereas at 1.5 mgl^{-1} it was difficult to distinguish the rodlet cells as the rodlets seem to disappear along with visibly reduced numbers of rodlet cells. This is possibly because of two reasons: firstly the connective tissue of the septal region becomes totally dominated by granular cells at this concentration of ammonia, visually giving the connective tissue an appearance of a granular tissue and secondly it appears that the rodlet cell numbers decline, possibly due to the destruction of rodlets as a consequence of high ammonia levels. Sometimes only the pear shaped cell borders without any contents were seen at this concentration of ammonia.

All the above changes were reproduced in the repeated experimental trial no. 2.

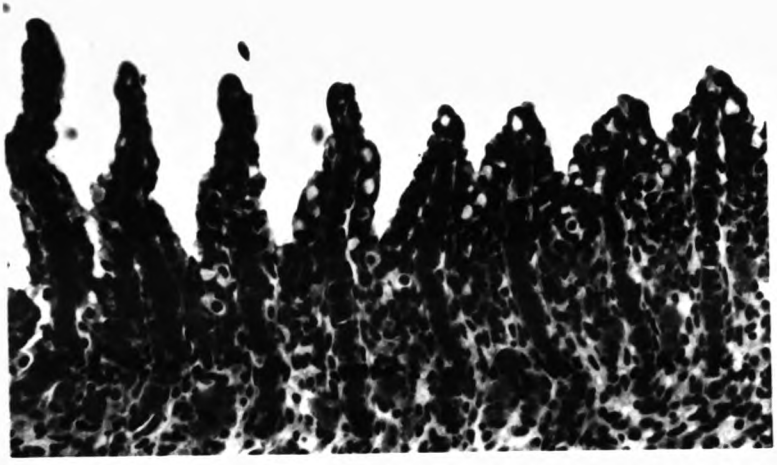
Telangiectasis was not a characteristic lesion in any of the fish examined, except in the case of a single fish sacrificed without any anaesthesia at the highest ammonia concentration

of 2.00 mgl^{-1} (Trial 2) after 36 hours of exposure (Plate 20a,b). These lesions consisted of significantly expanded lamellae filled with intact, well preserved blood cells, indicating that these telangiectic lamellae might have resulted due to the hyperactivity and thrashing movements of the fish at this high ammonia concentration, or as a result of the traumatic method of killing (Plate 20c).

Plate 16.

(a) A photomicrograph of the control carp gill from the tip of a filament showing severe hyperplasia after 3 days in buffered dilution water. Note an increased cellular activity as seen from the increased number of cell nuclei.(H&E, 600X.)

(b) A SEM of the control carp lamellae from the tip of a filament showing an upsurge of cells on to the lamellae from the filament epithelium (1000 X).



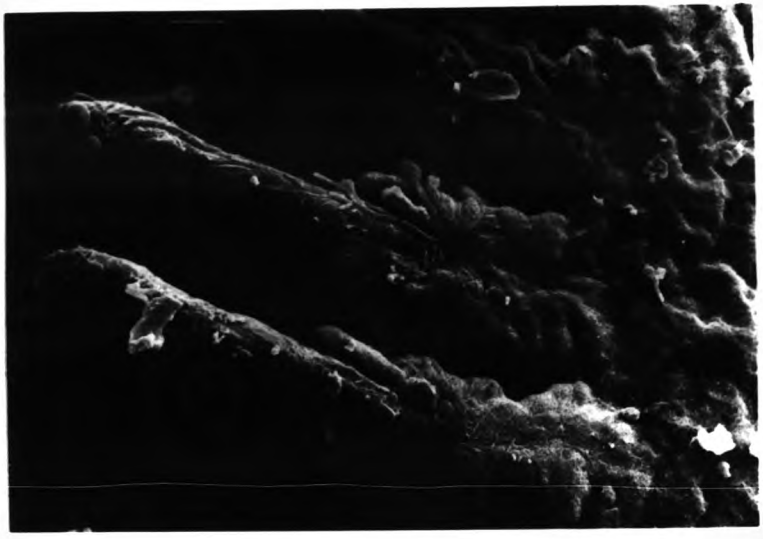
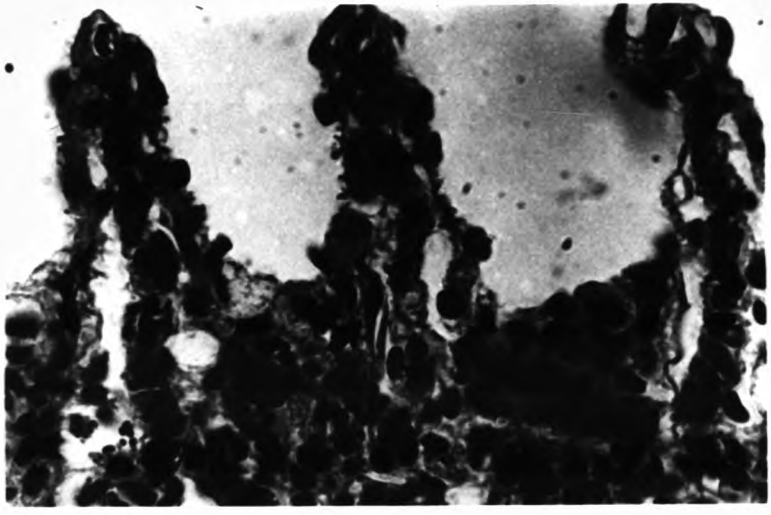


Plate 17.

(a) A photomicrograph of control carp gill lamellae after 3 days exposure in phosphate buffered dilution water. Note increased loosening of the lamellar epithelium and migration of fibroblast cells (arrowed) into the lamellar epithelium (H&E, 1500 X).

(b) A photomicrograph of control carp gill lamellae after 7 days of exposure in phosphate buffered dilution water. Note an increased lamellar oedema, infiltration of fibroblasts and their transformation into goblet shaped mucous cells (arrow head)(H&E, 1500 X)



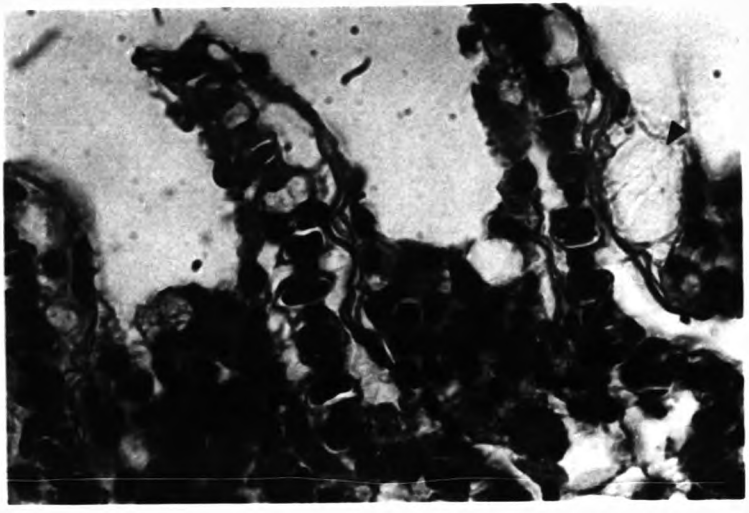
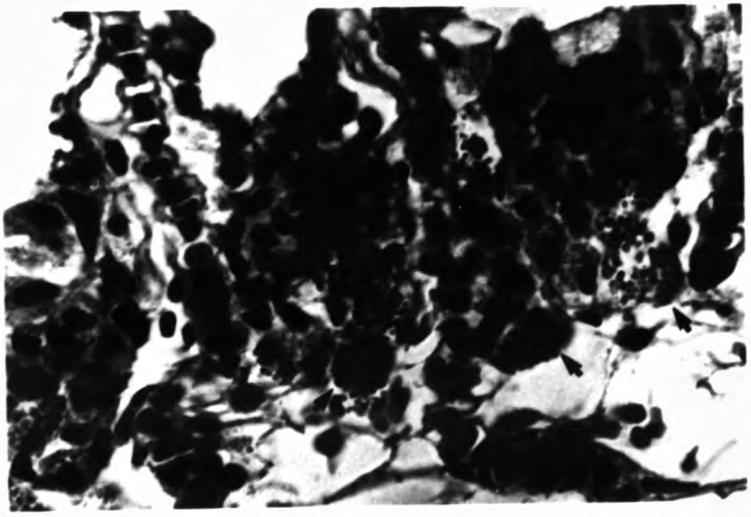


Plate 18.

(a) A photomicrograph of the control carp gills after 7 days in phosphate buffered dilution water, showing intense infiltration of the eosinophilic granular cells into the epithelium (arrowed) (H&E, 1500 X)

(b) A TEM of a similar area as above showing mostly intact (arrowed) or only partially degranulated (arrow head) EGCs. Note the increased cellular activity and oedematous changes in the interlamellar region 2800 X



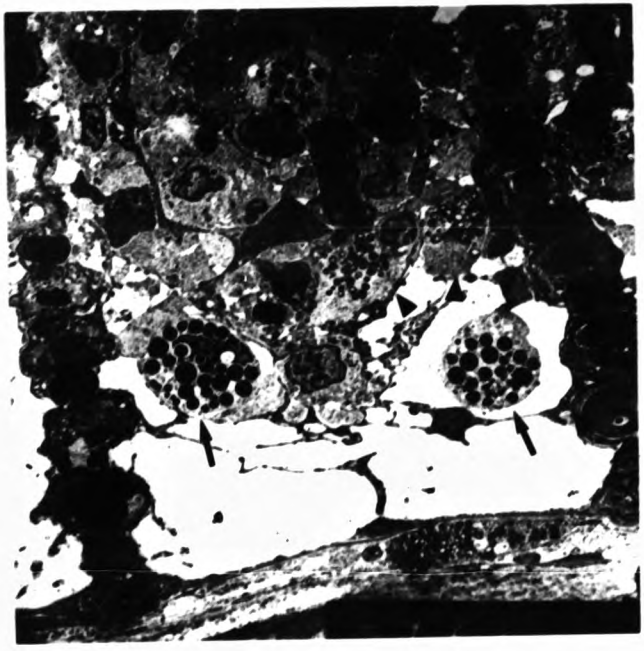


Plate 19.

(a) A photomicrograph of the septal region of gill from control carp showing relatively fewer rodlet cells (arrowed) (Methylene blue/Basic fuchsin - 600 X)

(b) A photomicrograph of the rodlet cells in the gill connective tissues of carp exposed to 1.5 mgl^{-1} UIA for 3 days. Note an increased density of the cells (Methylene blue /Basic fuchsin 1500 X)

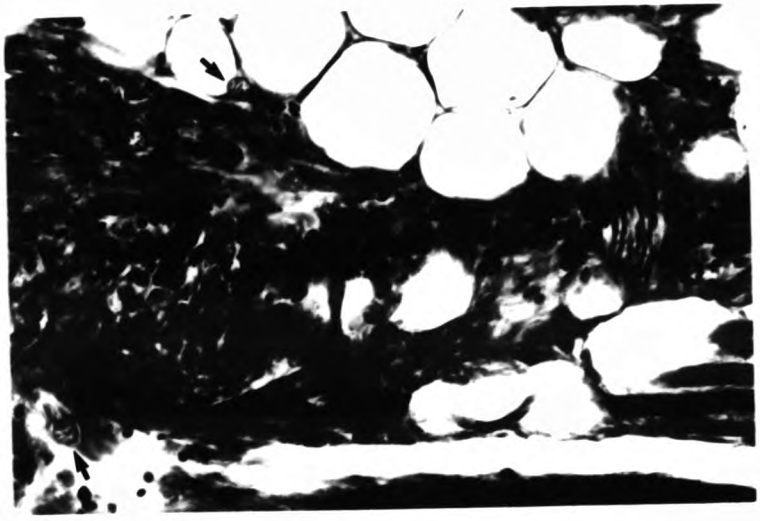
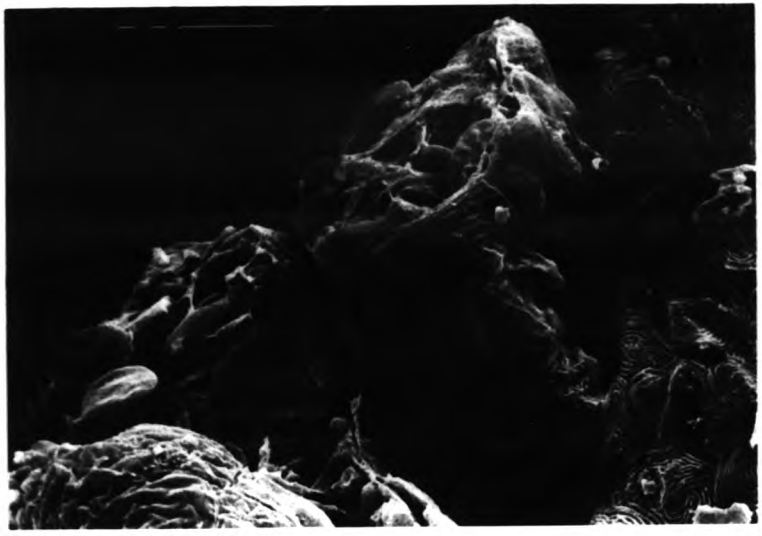


Plate 20.

a) A SEM of carp gill exposed to 2.0 mgl^{-1} UIA for 36 hours and then sacrificed without any anaesthesia. Note the development of lamellar telangiectasis on a few filaments. (600X)

(b)Telangiectic lamellae showing extensive damage to the lamellar epithelium. Note the ruptured lamellar epithelium (SEM, 700 X).

(c) A fractured telangiectic lamella showing the well preserved intact red blood cells, indicating that the lesion is recently formed 1700 X.



4.4. DISCUSSION

The results show that the tissue lesions characteristic of ammonia exposure occurred only in fish exposed to higher ammonia levels of 1.0 mgl^{-1} UIA (=35 mgl^{-1} TA) and 1.5 mgl^{-1} UIA (=52 mgl^{-1} TA), both after 3 and 7 days exposure. The absence of such lesions in fish exposed to 0.5 mgl^{-1} UIA (=18 mgl^{-1} TA) indicates that this ammonia concentration is not sufficient enough to ensure such pathological changes as seen at 1.0 mgl^{-1} or 1.5 mgl^{-1} UIA, and hence is relatively non toxic within the present experimental period of seven days.

However, in contrast to a "normal" gill appearance, the gills of the control fish showed severe structural changes which were not present in any of the ammonia exposed fish, or in the stock fish examined prior to their use in the acclimation and subsequent experiments. Although no such tissue changes seem to have been described in the literature, Kuhn and Koecke (1956) have noted the presence of fibroblast cells within the lamellae of the control or normal gills of their experimental goldfish while studying the effects of different pollutant substances using distilled water as dilution water. These authors have described such fibrocytes as a normal feature of fish gills. The histological illustrations of normal gills provided by these authors clearly indicate large numbers of fibrocytes similar to those seen in the current control fish, but only resembling early nontransformed stages. However, no such account of fibrocyte proliferation or presence has been reported in past descriptions of normal fish gills (Laurent and Dunel,1980; Hughes, 1984; Laurent, 1984). Neither is there any pathological account of the occurrence of fibrocytes or

similar cells in fish gill lamellae. The gills of the experimental fish of Kuhn and Koecke (1956) although containing fibrocytes, appeared to be overwhelmingly thickened due to an obvious hyperplasia of both filament and lamellar epithelium.

The failure of these authors to recognise pathological changes in control fish and the lack of information as to the source and maintenance of fish prior to their use in the experiments makes it impossible to speculate on the causes of such changes in their fish. In the current experiments the dilution water was buffered with 0.01 M phosphate buffer using di-sodium hydrogen orthophosphate and sodium di-hydrogen orthophosphate (Hasan and Macintosh, 1986).It is suspected that these buffer chemicals had some drastic effect on the physiological acid-base balance of the fish by contributing large amounts of external sodium and phosphate ions and thereby stimulating the fish gill tissue to react in such way as to thicken the filamental and lamellar epithelium to reduce the entry and possible effects of these ions. Since the lamellae are covered with a simple squamous epithelial layer, the increased number of intra-lamellar spaces and the infiltration of fibrocyte like cells may provide a protective barrier between lamellar blood sinuses and the external medium, as a possible compensatory mechanism, although further studies are needed to investigate this. It was difficult to precisely pinpoint the source of these fibrocyte like elongated cells histologically. Since they were only seen at the lamellar bases on either side of the pillar capillary network, they probably develop from the cells within the inter lamellar epithelium and move upwards by squeezing into the lamellar and inter-lamellar epithelial cell

spaces. As these cells move up they accumulate mucus vesicles towards their upper end, and appear like goblet shaped mucous cells, eventually opening to the exterior both in the interlamellar areas, and on the lamellae themselves, but still possessing an elongated tail like posterior part within which lies the elongated and/or compacted nuclei (Plate 24a,b). The possible role of such mucous cells could only be explained as a protective measure against the possible osmotic effect, and to barricade the loss or entry of ions into the gill epithelium. The other functions that have been ascribed to mucus, such as protection against parasites, bacteria or irritants such as ammonia or nitrite does not seem relevant under the circumstances.

Further, the appearance of extended lamellar oedematous spaces sometimes containing infiltrated cells seems to implicate a protective and osmoregulatory manifestation of the gills Hughes and Wright (1970), Hughes and Gray (1972) and Hughes (1978) have described the inter-cellular spaces in the secondary lamellae as lymphoid spaces, and as these spaces contained a variety of white blood cells, suggested that they might perform a function similar to those of the alveolar macrophages, and that the circulating fluid may be important in the protective and osmoregulatory function of the gills.

Severe hyperplasia noticed in the lamellar and filamental epithelium of the control gills once again seems to indicate a strong irritant reaction at the gill surface.

Although several workers have used various buffers in ammonia toxicity studies (Rice and Stokes, 1975; Robinette, 1976; Rubin

and Elmaraghy, 1977; Tomasso, *et al*, 1980; Hasan and Macintosh, 1986; Sheehan and Lewis, 1986) unfortunately none of these studies were specific histological investigations, and thus the possibility of any of these buffers having been detrimental to the fish gills seem to have proceeded unnoticed particularly so in the case of Hasan and Macintosh (1986). Even though several standard toxicity procedures recommend the use of buffers to regulate the pH of the water systems, no studies have been conducted to see the effect of buffers themselves on fish histology. Hasan and Macintosh (1986) investigating the lethal limits of ammonia to common carp fry under similar buffered dilution water as used in the present experiment noted a 3.1% mortality in their control fish which was equivalent to the mortality noticed at 0.43 mgl^{-1} UIA, but there were no mortalities at the low ammonia levels of 0.119 and 0.23 mgl^{-1} UIA in the same experiments. Although this may indicate that the mortality of the young fry in the control was most probably due to the buffer effect , no apparent ill effects were reported by these authors.

The transformation of fibrocyte-like cells into mucous cells as seen in this experiment has not been described before in fish gills or any other fish tissue, although the transformation of fibrocytes into lymphocysts in case of lymphocystis disease is well established. Fratantoni, Hall and Neufeld (1968) have described the transformation of fibrocytes into mucus-containing cells in patients suffering from Hurlers and Hunters disease syndrome, as the faulty degeneration of fibrocytes. This may further explain a possible potentiality of the mesenchymal cells of the interlamellar epithelium, or more probably the circulating blood leucocytes to give rise to

other cell types under such special circumstances. The transformation of blood leucocytes into fibroblasts has been very well established in higher animals (references cited by Stirling and Kakkar 1969).

The most interesting observation of the present investigation is that of the absence of tissue lesions in fish subjected to 0.5 mgl^{-1} UIA. No lesions as seen in control fish or fish subjected to the higher ammonia levels were observed, although a varying number of fibrocyte cells were seen in their early non transformed stages, probably representing those that were initiated and developed earlier at the time of acclimation only in the basal areas of the lamellae. They were not seen either migrating up into the lamellae or transforming into mucous cells, but only remaining at the basal areas of the lamellae, thus leaving the lamellae appearing perfectly normal except for the presence of fibrocyte cells. However, an increased mucous cell activity could be noticed as a generalised response to any low level toxicant, in addition to a moderate increase in chloride cell numbers in most of the fish exposed to 0.5 mgl^{-1} UIA.

The absence of tissue lesions in the epithelium of both filament and lamellae not only indicates that this concentration of ammonia is not toxic to carp, but more importantly that the presence of ammonium chloride at low levels may in fact be beneficial against the effect of buffer seen in the control fish. A possible equilibrium or compensatory state of ionic concentrations and the influx-outflux patterns may be working in a compatible way wherein the proper ionic /acid base balance is maintained due to the

presence of ammonium chloride.

However, these interesting aspects need further investigation to definitively answer the questions as to the precise mechanism of the development of lesions seen in the controls, and their absence in ammonia treated fish where in the cellular manifestations took a different form because of the problems faced by the fish in presence of both buffered water and ammonia. How the addition of low levels of ammonia to the buffered dilution water counteracts the effects of buffer needs to be further explained.

Cellular changes resulting in hyperplastic and hypertrophic epithelial swellings were only noticeable at 1.0 mgl^{-1} UIA, and the extent and the severity of these lesions drastically increased at 1.5 mgl^{-1} UIA both after three days and seven days of exposure, which is a clear indication of the toxic action of increased concentration of ammonia on the cellular structures.

Flis (1968a, 1968b), observed severe necrosis and disintegration of lamellar epithelium and lamellae in carp exposed to ammonia concentrations of 0.93-1.25 mgl^{-1} UIA over a 10 day experimental period. The pathology observed by these authors were very severe and included cell nuclei enlargement, vesiculation and disintegration, and the total loss of lamellar epithelium in some areas leaving the capillaries exposed. Cellular infiltration into the tissues and excessive mucus secretion was also noticed. Similar but more pronounced changes were also noticed by the same author after 35 days exposure of carp to ammonia concentrations of only 0.12 mgl^{-1} UIA.

However, in the present study, although severe epithelial lesions and necrosis were noticed, the lamellar epithelium was less affected in terms of lifting and its subsequent loss from the lamellae, as noticed by Flis, (1968a, 1968b). Kuhn and Koecke (1956) similarly found that the lamellar epithelium was least affected though other necrotic changes were identical to those found by Flis (1968a,1968b). The hypertrophic and hyperplastic nodular swellings seen in this study have not been reported before in carp. However Smith (1984) found massive hyperplastic lesions grossly visible on the heads of fathead minnows (Pimephales promelas ; Cyprinidae) exposed to high concentrations of ammonia (1.25 mgl^{-1} UIA) for 50 days. These lesions consisted of hyperplasia of the primitive meninx tissue surrounding the brain as a response to high ammonia exposure, and the severity of the lesions was positively correlated with the ammonia concentration. The lesions they observed consisted of a varied cell type in which many swollen cells containing small nuclei and foamy cytoplasm were seen along with rodlet cells which were diffusely scattered throughout although often concentrated at the periphery. These lesions observed by the above authors seem to resemble the kind of lesions seen in carp in the present experiments, but in a different organ. Since carp and minnows belong to the same family, possibly their tissue reactions and their ammonia tolerance capabilities may be similar, and this may further indicate a possibility of a phylogenetic variation as to the differences seen in the tissue reactions in different fish groups to ammonia.

The infiltration of eosinophilic granular cells has been described as a characteristic inflammatory reaction to both acute and sub acute ammonia toxic effect (Kovács-Gayer, 1984).

In the present study infiltration of strongly PAS positive eosinophilic granule cells into the gill epithelium was seen. Along with these EGCs, infiltrations of mononuclear cells that stained negatively for PAS were seen not only infiltrating the gill epithelium but being involved in the processes of the development of the lesions and necrosis. Although these EGCs have not been identified by the earlier workers in ammonia toxicity studies recent investigators have been recognising their presence and their possible role in initiating a inflammatory reaction in response to toxicant substances. Hornich and Tomanek (1983) reported the multiplication and infiltration of eosinophilic granule cells into the stratified epithelium of carp exposed to water with chlorinated lime as an inflammatory process. The same authors also reported that invasion by Trichodina and Chilodonella also induced such an inflammatory reaction. In the course of my own observations of diseased carp infected with monogeans or Ichthyophthirius and in carp suffering from Aeromonas infections, severe infiltrations of enlarged EGCs were noticed. Therefore it seems that these EGCs play some important role in the defence mechanism of the fish against infectious agents, or in alleviating the toxic effects of chemical pollutant substances including ammonia. In the case of fish exposed to high ammonia concentrations the EGCs became vacuolar due to the loss of the contents of the granules, whereas in the control fish although the numbers of EGCs increased possibly due to the effect of the buffer, only a few cells in the vicinity of cartilage showed partial vacuolation of granules, whereas the cells with in the epithelium retained intact granules. This indicates a possible detoxifying, cell lysing, or osmoregulatory role of the contents of these granules.

However, the most pronounced effect of ammonia on the gills was the proliferation of active and hypertrophic chloride cells on the lamellae, although their numbers also increased on the filament and interlamellar epithelium. High ambient unionised ammonia concentrations in water not only causes a passive influx of NH_3, resulting in increased plasma and intracellular ammonia concentrations, but can stop the passive efflux of ammonia from the fish (Hampson, 1976; Cameron and Heisler, 1983). Under such circumstances an alternative pathway of excreting ammonia by way of an active monovalent cation exchange transport system involving Na^+ and NH_4^+ has been suggested (Colt and Armstrong,1981; Cameron and Heisler,1983; Randall and Wright, 1987) based on the earlier works (Maetz 1973; Garcia-Romeu 1964; Girard and Payan 1980) that have demonstrated the presence of a Na^+/NH_4^+ Na^+/H^+ exchange system in the gills, and the ability of NH_4^+ to exchange for Na^+ absorption implicating that this process may significantly contribute to ammonia excretion. It is also known that at normal blood pH levels almost 99% of the ammonia exists as ionised ammonia (NH_4^+). Therefore the exaggerated chloride cell proliferation seen at 1.0 and 1.5 mgl^{-1} UIA is more likely to be a direct response to the need to eject NH_4^+ from the blood resulting from the passive influx of external environmental ammonia in its molecular form (NH_3) along the water/blood ammonia gradient, and then being trapped as NH_4^+ in the blood and body fluids due to their relatively low pH environment. Thus an alternative mechanism of excreting ammonia against the diffusion gradient via the gill Na^+/K^+ ATPase seems to be in effect, as has been suggested (Cameron and Heisler 1983; Stewart 1983) under high external ammonia conditions as a

result of elevated blood NH_4^+ and prevention of these methods of ammonia excretion.

As long as the environmental ammonia remains higher a passive diffusion of the unionized fraction of ammonia into the fish is inevitable. Once in the blood a portion of it could be converted to NH_4^+ thereby maintaining a constant level of NH_4^+ in the blood, and hence a constant pressure on the chloride cells across the gill epithelium for the active transportation in exchange for external Na^+ from the environment.

However, continuation of such a single exchange process without a stop would result in the build up of Na^+ ions in the cells and body fluids. In the face of such a build up of cellular and blood sodium concentration, it is suggested that a simultaneous Na^+/H^+ pump may operate to eliminate excessive Na^+ from the cells and body fluids in exchange for environmental H^+. Although such a Na^+/H^+ pump is expected to load the cells and body fluid with excess H^+ ions, it will more likely be beneficially utilized for the hydration of constantly influxing NH_3 as more and more NH_4^+ is actively pumped out in exchange for environmental Na^+. When both of these exchange processes are involved simultaneously neither Na^+ nor H^+ could be expected to accumulate in the cells or body fluids and thus a normal internal fluid pH (hydrogen ion concentration) and osmotic balance may be effected. A hydrogen ion shuttle inwards would otherwise tend to lower the blood pH, while a loss of blood or cellular H^+ ions would tend to increase the pH, and a constant Na^+ ion shuttle inwards may tend to alter the osmotic balance.

However, under extreme sublethal ammonia concentrations such as in the present experiment, the longevity of this ATP draining Na^+/NH_4^+ exchange process may not be sustainable for long, and a progressive loss of control of cellular and tissue physiological functions may ensue, as ammonia has been known to have both a membrane and metabolic effect (Campbell,1973) Subsequent degradation and death, as indicated by a range of cellular changes and necrotic processes in the current study may further indicate the failure of the chloride cells in their relentless effort to pump out NH_4^+ and at the same time other necessary ions leading to eventual poisoning by ammonia.

Although hypertrophy of the gill and lamellar epithelium has been consistently reported as the characteristic lesion in ammonia exposed fish (Flis, 1968a,b; Smith and Piper, 1975; Smart, 1976; Thurston, Russo and Smith, 1978; Cruz and Enriquez, 1982; Stewart, 1983; Thurston, *et al.* 1984) unfortunately no studies have identified the discrete cell types involved in this reaction. In the present investigation chloride cells have been identified as the principal cell type involved in the hypertrophic reaction, at the same time highlighting the possible importance of these cells in ammonia excretion.

However, such chloride cell proliferation and necrosis has been noted on the secondary lamellae in fish exposed to acid water conditions (Leino and McCormick, 1984; Leino *et al.* 1987), toxic heavy metal poisoning such as aluminium and acid (Karlsson-Norrgren *et al.*, 1986a; 1986b; Evans *et al.*, 1988), zinc (Matthiessen and Brafield, 1973; Crespo *et al.*, 1981; Crespo and Sala, 1986) and cadmium (Oronsaye and Brafield, 1984; Karlsson-

Norrgren *et al.*, 1985).

Similar chloride cell proliferation has also been noted in fish exposed to deionized or distilled water, and under conditions of tissue damage due to skin wounds or parasitic gill damage (Laurent and Dunel, 1980; Mclay, 1985), clearly indicating that the water quality conditions that result in ion loading or ion losses are the chief causes responsible for chloride cell proliferation, the necrosis of chloride cells being directly related to toxicant effect on them.

Histochemically, increased mucous cell activity was obviously a conspicuous reaction increasing ammonia concentrations, but it was not possible to make a more meaningful comparison between experimental ammonia treated fish and the reference control fish due to the obvious difficulties faced in the present experiment.

In the control fish, apart from the extensive proliferation of elongated mucous cells on the lamellae, the gill epithelium in general reacted drastically, resulting in a mucous cell layer unusually thicker than the ammonia treated fish (Plate 21). The lamellar mucous cells that were of specialized elongated type were restricted in their distribution only to the lamellae and inter-lamellar region, and were not seen either in the filament epithelium or in the epithelium that covers the gill arch and rakers. All other mucous cells were of normal ovoid or spherical shape, characteristic of the general carp gill epithelium. A profuse mucus secretion was evident both on the lamellae and the filament epithelium in the control fish. Sometimes the overwhelming proliferation of the specialised

goblet shaped mucous cells resulted in extensive copious mucus production on the secondary lamellae (Plate 22a and 23a) often covering the whole length of the secondary lamellae . Such goblet shaped mucous cells stained strongly positive for alcian blue stain at pH 2.5 and continued to do so at pH 1.0, indicating the predominance of sulphated mucus substances (Plate 23b). The mucus from the adjacent lamellae sometimes coalesced resulting in the sticking together of the neighbouring lamellae (Plate 23b), thus giving an appearance of false lamellar fusion in histological preparations. Under transmission electron microscopy the goblet shaped elongated mucous cells consisted of well packed discrete electron lucent membrane bound mucus vesicles (Plate 24a,b), whereas the other mucous cells of the filament epithelium contained a mixture of both electron lucent and electron dense vesicles (Plate 26a), indicative of the differences in their chemical composition.

The significance of this proliferation and migration of the specialized mucous cells on to the secondary lamellae is uncertain, but is most probably a protective response to the phosphate buffer, as the stock fish examined did not exhibit any such alterations and consisted of normal mucous cell and lamellar patterns.

Under normal circumstances mucous cells are generally absent on carp or trout gill lamellae, however, excess mucus extruded from the mucous cells of the primary epithelium might provide a continuous protective film of mucus to the secondary lamellae under instances of infection or irritation. The microridges on the gill epithelium (which are also present on the secondary lamellae of some species of fish such as trout) known to help

in anchoring such a mucus layer to the epithelium (Hughes and Wright 1970). Unlike trout gill lamellae, carp gill lamellar epithelial cells lack the microridges, which may otherwise perform such a function by holding the mucus flowing from the filament mucous cells. In addition to this disadvantage, the fact that the lamellar epithelium in general lacks the germinative basal layer that may give rise to normal mucous cell populations, and in the present situation, a possible necessity for a mucus layer to protect the respiratory lamellae against the unknown impact of the buffer, might have been the cause of such an extreme mucous cell proliferation and migration on to the secondary lamellae.

As already mentioned, the use of buffers in ammonia toxicity experiments to maintain constant pH levels is increasing, and in the light of the present experimental results it may well be necessary to verify the validity of the use of such buffers. An evaluation of the most commonly used buffers in the toxicity studies may be important in furthering their general use in toxicological studies.

In the case of fish exposed to 0.5, 1.0 and 1.5 mgl^{-1} UIA, although the mucous cell density was variable within the same treatment, and within the same gill between different areas, an increased mucous cell activity could be seen with increasing concentrations of ammonia, based on extensive histological observations. No mucous cells could be seen on the lamellae in the case of the fish exposed to ammonia. The increased mucous cell activity was also associated with an increase in size.

Morphologically, as observed with the scanning electron microscope, three different stages of superficial mucous cell secretions could be noticed in fish exposed to different ammonia concentrations. In fish exposed to 0.5 mgl^{-1} UIA, the external openings remained normal with occasional traces of mucus at the openings (Plate 12). The mucous cell openings in the case of 1.0 and 1.5 mgl^{-1} UIA were more distorted and a majority of the cell openings at 1.0 mgl^{-1} UIA had their openings plugged with thick mucus of a non-copious nature (Plate 13). At 1.5 mgl^{-1} UIA, such cells hardly retained the mucous plugs and a majority of such openings had distorted openings lacking any mucus plugs (Plate 14).

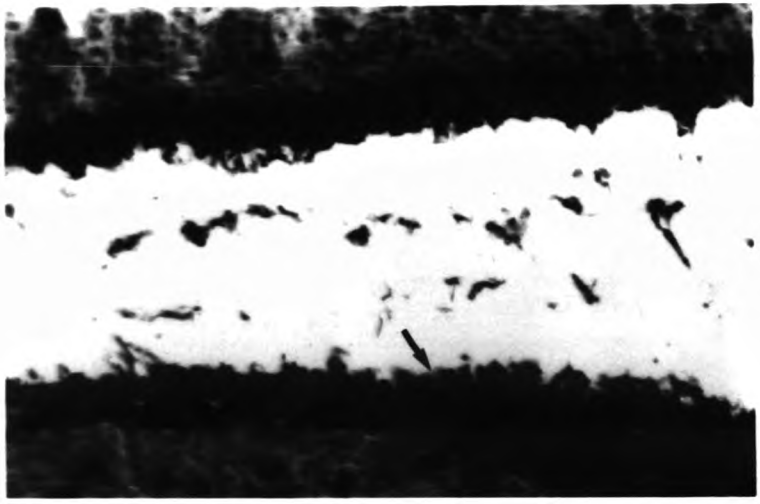
The mucous cells appeared exhausted in the control fish and their external openings were similar to those of the high ammonia exposed fish, but no thick mucus plugs could be seen in such cells indicating that they were secreting a copious mucus as was obvious from the thin mucus layer observed in some areas under the scanning electron microscope (Plate 25a,b).

Ultrastructurally the mucous cells at high ammonia concentration (1.5 mgl^{-1}) showed necrotic changes (Plates 26b,and 27a,b).The integrity of the individual mucus vesicles of the mucous cells were broken before the cells could reach the surface, resulting in small aggregations of mucus debris and vacuoles within such cells. Such changes could also be seen in the surface mucous cells, which was a clear indication of necrosis of mucous cells as a result of ammonia poisoning.

The other pathological changes of significant importance noted in the epithelium in general such as the condensed

cells,and cells under going pyknosis and other nuclear changes (Plates 15,26b,28), appears to due to the direct effect of ammonia on the cellular metabolism. Necrotic changes noticed in the epithelial cell such as the vacuolations in the cytoplasm,and the appearance of dark inclusions (Plate 29), seems to be due the interference of ammonia in the metabolism of cellular organelles (Campbell,1973 ; Chow and Pond,1972).

Plate 21. Photomicrographs of the control carp gill filaments after seven days showing severe proliferation of the mucous cells (normal type) on the filament epithelium.(PAS, 600X)



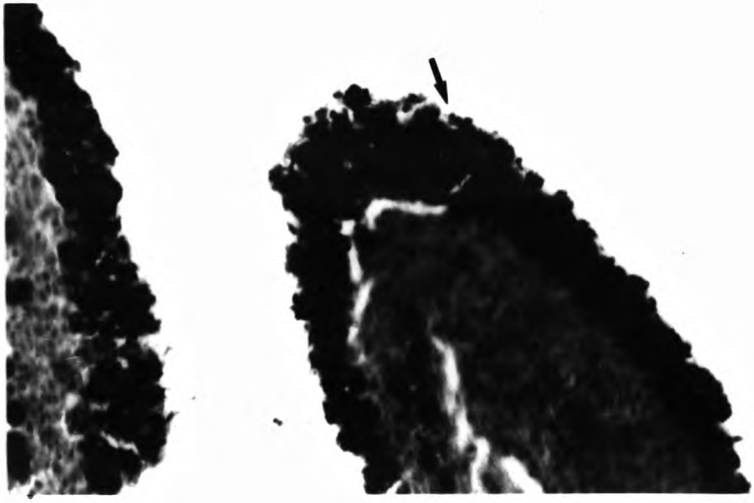


Plate 22.

(a) Photomicrograph from the transverse section of the control carp gill filaments after seven days exposure exhibiting lamellae secreting profuse mucus, covering almost the entire length of the lamellae (arrowed)(PAS, 600X)

(b) Photomicrograph of a control carp gill section stained with combined Alcian Blue and PAS stain at pH 1.0 after seven days exposure. Note the strong affinity of the goblet shaped specialized mucous cells to Alcian Blue while the EGCs are strongly PAS positive(arrowed). (PAS/AB pH 1.0, 1500X)

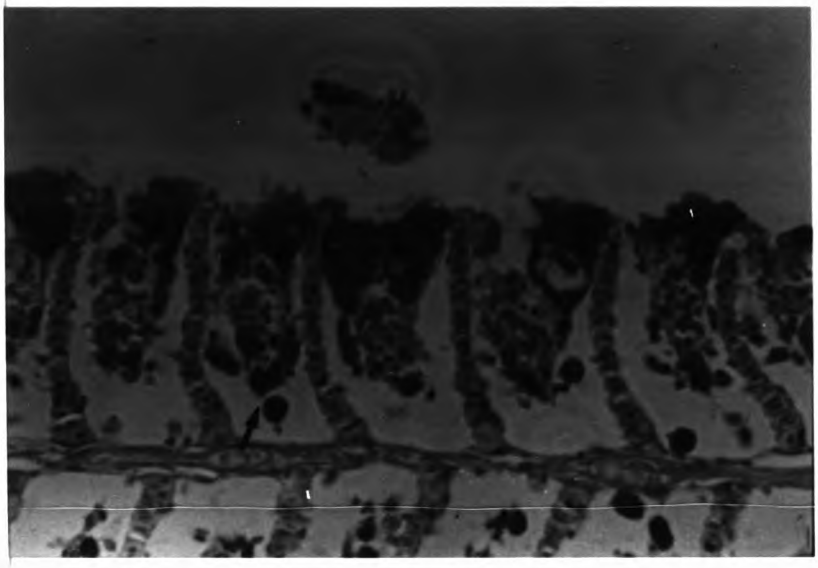
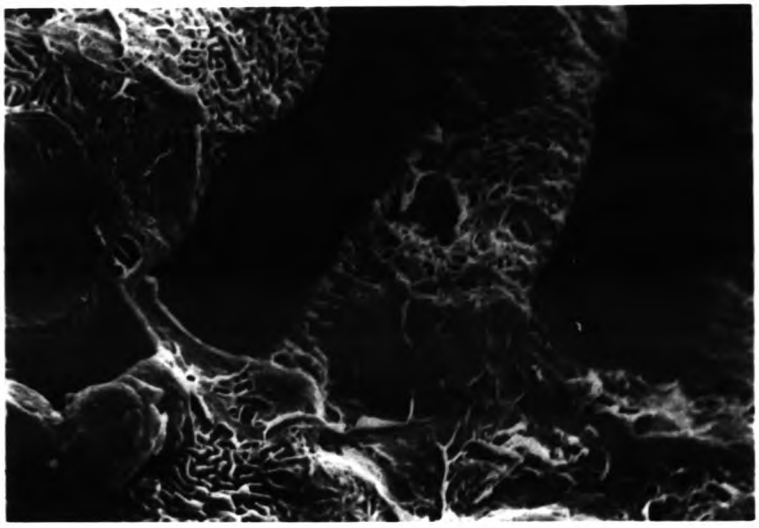


Plate 23. SEM of the lamellae from the control carp after seven days in phosphate buffered dilution water.

(a) Note a thin mucus layer on the lamellae probably produced by the lamellar mucous cells. 4500X

(b)Occasional adhesion sticking of the adjacent lamellae due to an increased mucus production on the lamellae. Note specialized mucous cells in the process of mucus secretion on one of the lamellae (arrowed) (SEM, 2300X)



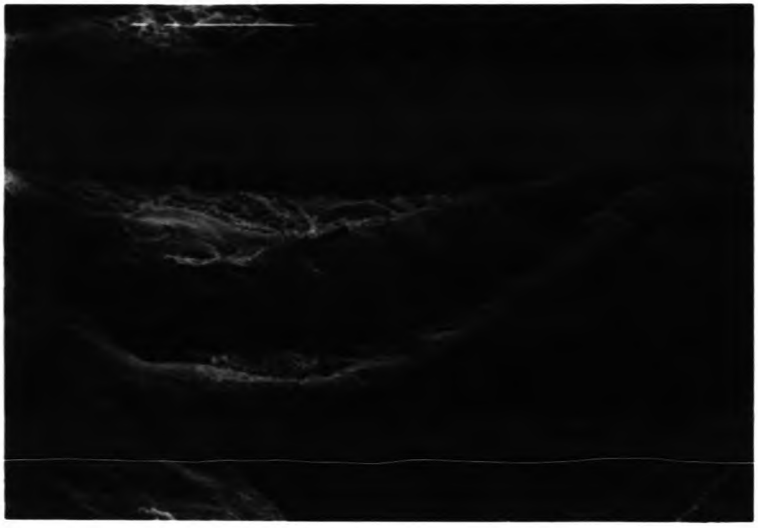


Plate 24.

TEM of the lamellae from the control carp gills after seven days.

(a) Note the migration of cells that look like fibroblasts (arrowed) and cells in the process of accumulating mucus vesicles as they ascend up into the lamellar epithelium (arrow heads) (4546X).

(b) A fully transformed mucous cell opening to the exterior (arrowed) with its tail end enclosing the nuclei. Note the predominance of the electron lucent mucous vesicles within these mucous cells. (4741X)

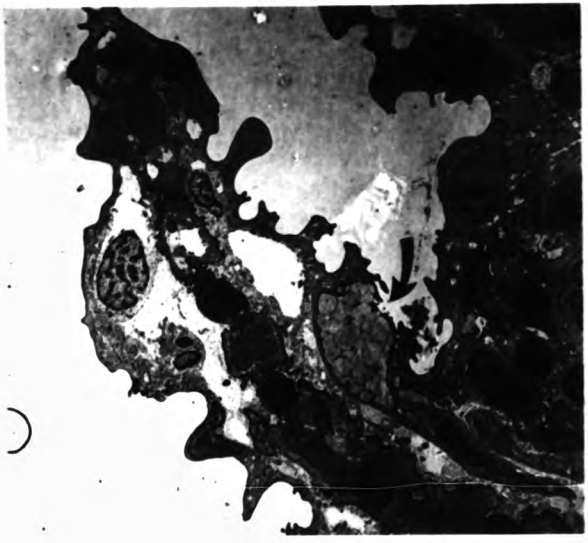


Plate 25. SEM of the mucous cell reaction on the filament epithelium of the control carp examined after seven days of exposure in phosphate buffered dilution water.

(a) Increased mucous cell activity is indicated by the appearance of numerous mucous cell openings on the filament epithelium, and their exhaustion (SEM, 120X)

(b) At higher magnification the epithelium appears unevenly shrunken, probably due to the exhaustion of numerous mucous cells just below the Malphigian cells (SEM, 850X).

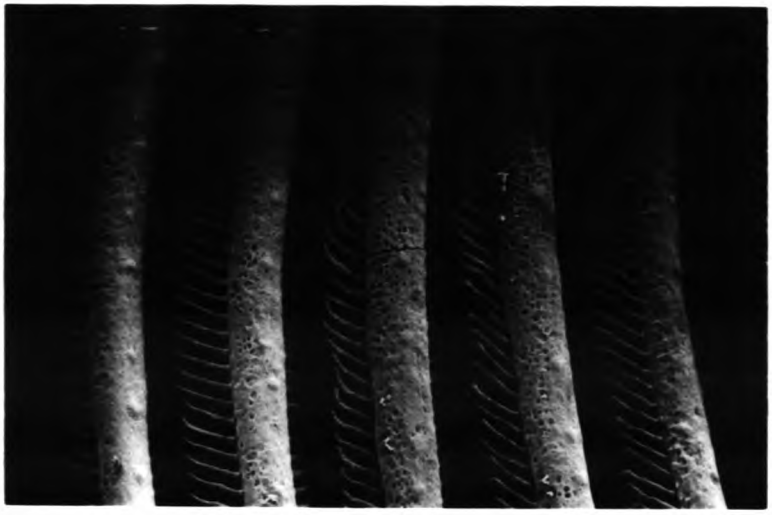
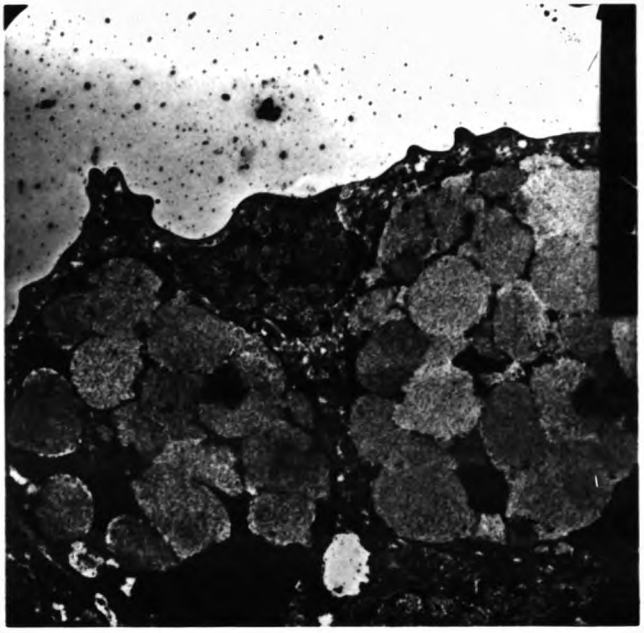


Plate 26.

a) TEM of two adjacent mucous cells from the control carp gill filaments after seven days exposure, showing normal mucus vesicles with a predominance of electron lucent vesicles and a few electron dense vesicles (7640X).

(b) TEM of the gill filament epithelia from carp exposed to a high concentration of ammonia (1.5 mgl^{-1} UIA) for seven days showing the occurrence of more electron dense mucus vesicles and a low density of electron lucent vesicles. Note the condensation of a necrotic mucous cell (big arrow) and a focal necrosis of mucus vesicles (small arrow) (4800X).



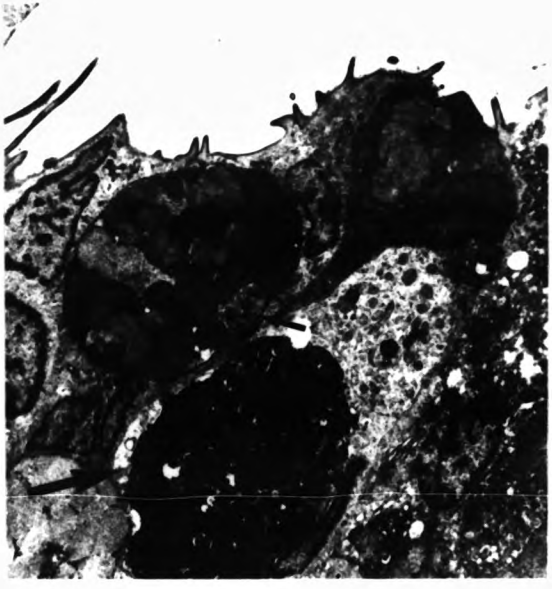
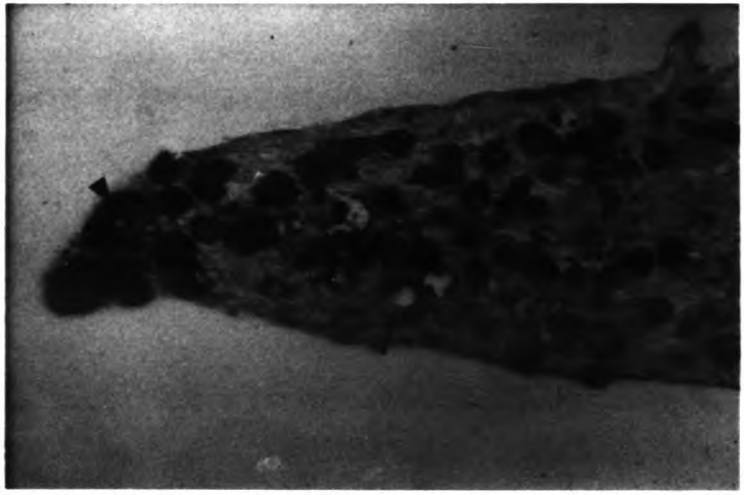


Plate 27.

(a) A photomicrograph of the carp gill epithelium from the tip of a filament exposed to 1.5 mgl^{-1} UIA for seven days. Note vacuolation (small arrows)and clumping of the mucous cell contents (arrow heads) (PAS, 600X)

(b) TEM of necrotic mucous cell from carp gill epithelium after seven days exposure to 1.5 mgl^{-1} UIA. Note the clumping of mucus vesicles and their flocculant contents (arrowed) along with normal looking electron lucent mucus vesicles (13760X)



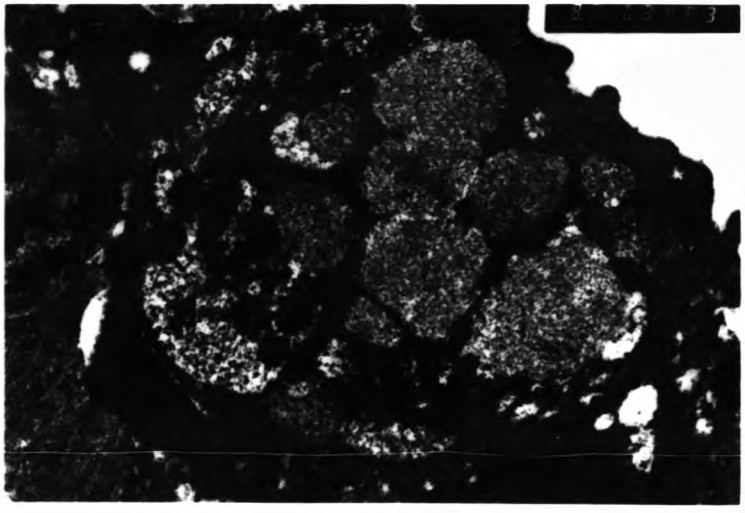
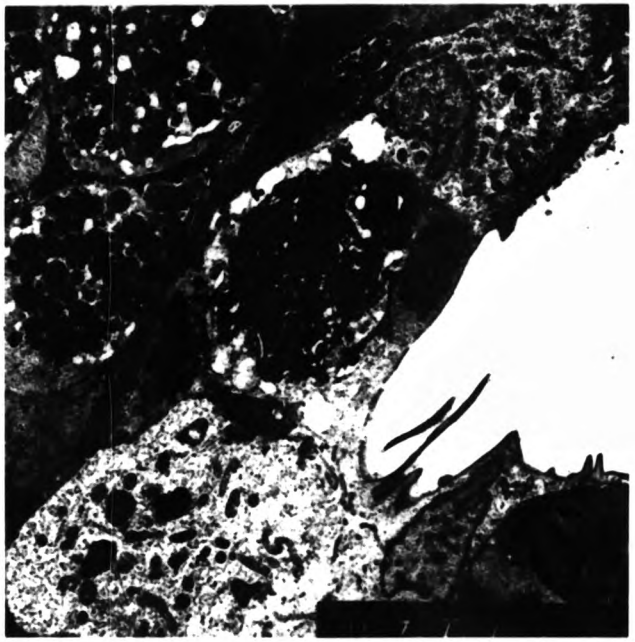


Plate 28.

(a) SEM of the lamellae from carp exposed to 1.5 mgl^{-1} UIA showing a necrotic cell within the lamellar epithelium. Note the normal nuclei of a non differentiated cell while the adjacent cell shows breakdown and lysis of the nucleus (arrowed). Also note the normal pillar cell (PC) and the red blood cell (RBC) (7692X).

(b) SEM of a filament epithelium from carp exposed to UIA concentrations of 1.5 mgl^{-1} for seven days showing a condensed, darkened necrotic cell with hardly any identifiable subcellular structures. Also present is a necrotic chloride cell, normal and degranulating EGCs (4,850X)




PC
RBC

Plate 29.

SEM of an epithelial (Malpighian) cell undergoing necrotic changes in the Cytoplasm . Note the normal nuclei, increased number of Cytoplasmic vacuoles, dark inclusion bodies and the reduction in the microridge hight (arrowed). 4900X

The rodlet cells that were noted in the present experimental carp gills have also been described by various workers in different organs of fish including gills, kidney, intestine olfactory and pharyngeal epithelia, bulbus arteriosus, heart, liver, gonads, menings, brain, spinal chord, and eye (Bannister, 1966; Leino, 1974; Desser and Lester, 1975; Flood, Nigrelli and Gennaro, 1975; Mattey, Morgan and Wright, 1979; Karlsson, 1983; Smith, 1984; Martinez, 1987). However, their distribution with respect to sites and numbers to vary between and within species, often according to season, crowding or ionic concentration of the water (Leino, 1974).Although the precise nature and function of such cells is still obscure, the controversy as to whether these cells are a type of parasite initially described as Rhabdospora thelohani. by Laguesse (1906; cited by Bannister 1966) who believed them to be the sporocyst stages of a sporozoan parasite, as later supported by Bannister (1966), or a type of epithelial, endothelial or connective tissue cell as suggested by later workers, remains unresolved.

However, the apparent lack of host and tissue specificity of the rodlet cells, and the fact that these cells were present in the gills of swordtails even immediately after their birth (Leino,1974) suggested that they are not parasitic. Such a view has been upheld by later workers such as Desser *et al.*, (1975) and Mattey *et al.*, (1979) based on their extensive electron microscopical studies dealing with the structure and development of rodlet cells in two different fish species. Since these cells are usually found in tissues such as epithelium (gill and intestinal) & endothelium, sites suited

for secretion into blood, body cavities or external milieu, they have been implicated in an osmoregulatory function (Mattey *et al.*, 1979). Under the present experimental conditions the rodlet cells visibly increased in the fish exposed to ammonia, indicating a probable osmotic role as suggested by Mattey *et al.* (1979). However, the loss of the rodlets at 1.0 mgl^{-1} ammonia concentration and the disappearance of these cells at 1.5 mgl^{-1} UIA concentration, may indicate a release of the contents of the cell and their subsequent loss as a consequence of the effect of high ammonia. Mattey *et al.* (1979), based on the observation that the rodlet cells were virtually absent when the fish came from dilute sea water as compared to those that came from fresh water, indicated that changes in osmotic pressure may cause secretion of rodlet sacks or the loss of the whole cell.

Unlike the present experimental observations of the rodlet cells of carp, the rodlet cells from the epithelial tissues of, Dicentrarchus labrax (L) stain positive for PAS staining. Therefore Mattey *et al.* (1979) suggested that they may also have functions similar, or complimentary to, mucous cells as suggested by Leino (1974).

Since in the present experiments the rodlets were only seen in various connective tissues of the gill arch that supported the blood vessels, and that of tissues of the septal region, and not in the filament or lamellar epithelium this may suggest that their secretions might play an osmotic role rather than that to do with the mucus.

Smith (1984) who reported severe hyperplastic swellings in the primitive meninx of fathead minnows exposed to extremely toxic levels of ammonia also noted an increased number of rodlet cells in these brain lesions, which once again indicates a possible role of these cells in ammonia toxicity. Further experiments will be necessary to throw more light on these aspects.

CHAPTER 5

EFFECT OF AMMONIA ON TROUT GILL PATHOLOGY

5.1. INTRODUCTION

The purpose of this study as already outlined in chapters 1 & 2, was to examine the effects of ammonia at both near lethal and sublethal concentrations on the gill pathology of trout and to provide a comparison with those of the lesion types that have already been described by various workers. These experiments were also aimed at providing a comparative account of the gill pathology in trout with respect to the changes already noted in the carp experiments (Chapter 4).

5.2. MATERIALS AND METHODS

Initially young rainbow trout, Salmo gairdneri (Richardson), destined to be used in ammonia exposure experiments were obtained from a commercial trout hatchery after prior histological examination and confirmation of normal gill structure. These fish were maintained in 1 m^2 fibreglass tanks in an existing water flow through fish holding facility receiving water from a public tap water supply at ambient temperature conditions. The fish were fed with commercial trout pellets twice daily.

Prior to the experimentation and after a month's maintenance in this system, a sample of 10 fish were processed for histological and scanning electron microscopical examination so as to obtain an initial assessment of the stock fish gills. These observations revealed that the fish suffered from severe lamellar fusions, hyperplasia and hypertrophy (Plates 30a,b, c and d). Maintenance of the fish in the same system for a further one month did not lead to any improvement in the gill structure, and the lesions were consistently present.

Plate 30.

SEM of trout gills maintained in a water flowthrough system receiving tap water as the main supply for a period of one month.

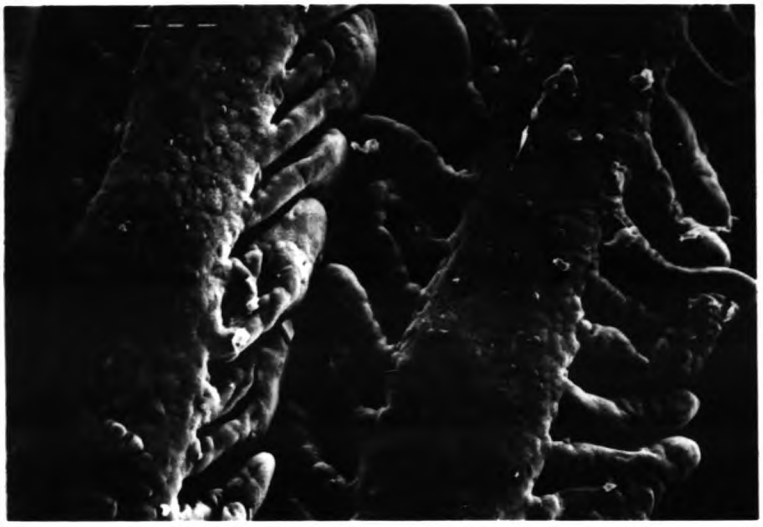
(a) Severe hyperplasia and fusion of the lamellae. 350X

(b) A higher magnification view of the fused lamellae showing the perfect fusion of the lamellar epithelium. 1800X

(c) Severe lamellar fusion resulting in the fusion of a series of lamellae. 1050X

(d) SEM of the severe hypertrophy noted in the same fish after one month. 1200X

a



b

c

d

The water quality parameters measured such as ammonia and nitrite, remained well below the recommended safe limits. The dissolved oxygen levels varied between 7.5-8.7 mgl^{-1}, but considerable fluctuations in pH were noted during this period varying between 5.5 and 6.8. Occasional mortality of one or two fish in some tanks could not be attributed to any parasitic infections.

However, from enquiries made of the Water Board it was suspected that the aluminium-based chemicals used in the water treatment were the possible cause of these gill alterations, and at the same time responsible for the reduction in water pH, although the presence of chlorine compounds normally used in the water treatment was not rejected as another possible factor leading to gill hyperplasia (Mitchell and Cech 1983).

Whatever the ultimate cause of these gill alterations shown by histological and scanning electron microscopical observations, it was decided that the public water source was unsuitable for either holding fish, or for the purpose of experimentation.

Thereafter for all experimental purposes, the fish were obtained only a week prior to experimentation, and were directly transferred into the acclimation tanks containing synthetic dilution water.

5.2.1 EXPERIMENTAL ANIMALS

The source of experimental trout and their maintenance prior to experimentation are detailed in chapter 3.

Young rainbow trout used throughout these ammonia toxicity

experiments came from different trout hatcheries in Scotland, as and when required. Fish from a single stock were used at any one time in a particular experiment to maintain a uniform population in all the treatments.Two experimental trials; trial 1 and trial 2, were carried out independently using two different fish stocks at two different periods of the year. Fish weighing 14.36 grams (± SD, 5.15) and measuring 12.01 cms (± SD, 2.31) were used in the first trial, whereas the trout used in the second trial weighed 16.03 grams (± SD, 3.00) and measured 11.92 cms (± SD, 0.64).

The objectives of these experiments were as set out in chapter 1 and with similar experimental procedures as outlined in chapter 4 for carp, to investigate the effects of ammonia on trout gills, and to provide a comparative description of the changes in the trout gills to those seen in the carp experimental trials. Changes that were made in the experimental conditions were those of temperature and hardness. Unlike carp, trout being a cold water fish, a temperature value appropriate to this species was adopted, which is usually 15 ± 2°C (APHA, 1980). Since the trout obtained from the commercial trout hatcheries were reared and maintained at a water hardness of approximately 25 ppm as $CaCO_3$, typical of Scottish riverine waters, a similar hardness water was adopted for use in these trials. A 25 ppm hard synthetic dilution water was made up by mixing appropriate amounts of dilution water stock solutions in deionized distilled water as outlined in chapter 4. The use of a water of 25 ppm hardness or 250 ppm hardness has been recommended for experiments with rainbow trout by the Ministry of Housing and Local Government (1969).

5.2.2 EXPERIMENTAL SYSTEM

The experimental system consisted of static glass tanks of specifications provided in chapter 4, with similar water holding capacity, each tank being used to hold 85 litres of dilution water. The experimental setup was identical to the carp experiments except that no submersible heating coils were required, as all the tanks were maintained in a constant temperature room where the water temperature remained constant at 15°C.

5.2.3 TEST WATER

The dilution water used was of 25 mgl^{-1} (or ppm) hardness as $CaCO_3$.To provide constant pH throughout the experimental period, the dilution water in all the experimental tanks was buffered with 0.01 M phosphate buffer by addition of appropriate amounts of di-sodium hydrogen orthophosphate and sodium di-hydrogen orthophosphate.

5.2.4 ACCLIMATION AND EXPERIMENTAL PROCEDURE

For the reasons indicated earlier, soon after arrival the trout were transferred into acclimation tanks containing synthetic dilution water and there was no intermediate holding in the tap water. Fish were acclimatized to 25 ppm hard dilution water for 6 days,and the water in these tanks was partially changed every day with freshly prepared ammonia free dilution water. Twenty four hours prior to the experimentation, all the experimental fish were also acclimatized to buffered dilution water at the same concentration as used in the experimental tanks.

5.2.5 AMMONIA CONCENTRATIONS TESTED

In the experimental trial 1, two ammonia concentrations at 0.23 mgl^{-1} UIA and 0.31 mgl^{-1} UIA were tested along with one control treatment. As the reactions of the fish noted in the experimental trial 1 were relatively mild, a second trial was carried out under identical experimental conditions but at elevated ammonia concentrations. The second trial was aimed at extending the results of trial 1. The concentrations of ammonia tested in the second trial were 0.43 mgl^{-1} UIA and 0.57 mgl^{-1} UIA, with one control treatment.

5.2.6 REPLICATION

At all the concentrations of ammonia tested, a duplicate set of tanks was used. Similarly in both the trials a duplicate set of control tanks was used.

5.2.7 NUMBER OF FISH AND THEIR DISTRIBUTION

Ten fish were used in each experimental tank and hence twenty fish tested at each concentration. Fish were randomly distributed to the experimental tanks. Fish were netted with soft meshed shallow nets, taking care not to chase the fish.

5.2.8 EXPERIMENTAL DURATION AND SAMPLING INTERVALS

Each experimental trial was carried out for 7 full days, 50% of the fish in each tank being sampled at the end of day three,

and the rest at the end of day seven. Periodical observations of the behaviour of fish were made and the mortality of the fish was noted as and when it occurred.

5.2.9 MEASUREMENT AND ANALYSIS OF WATER QUALITY

The methods of measurement of water quality parameters were as outlined in chapter 3. The frequency of measurement of various water quality parameters such as ammonia, nitrite, pH, temperature, hardness, alkalinity, and conductivity were similar to those in chapter 4.

5.2.10 TISSUE SAMPLING

The first two gill arches from any one side of each fish sampled from a treatment tank were fixed together for electron microscopic studies. Thus there were two groups of tissues, comprising all the first and second gill arches of the fish sampled on day three and all the first and second gill arches of the fish sampled on day seven. A sub-sample of 3 to 4 halved or whole gill arches were used for SEM, whereas 3 to 4 small areas from each of these groups were used for blocking out for TEM studies. For light microscopic histological studies, 4-6 gills from each fish sampled, both on day 3 and day 7 were processed and sectioned separately. This enabled the examination of each fish separately at both sampling periods.

Since histological examination was extensive, only a small number of sub samples were examined using electron microscopy. The method of fish anaesthetization and the procedures followed for fixation and processing of tissues for both electron

microscopy and light microscopy wax embedding are as outlined in chapter 3.

All the gills fixed and processed for wax embedding were sectioned and stained with various stains for both general observation and for the histochemical differentiation of mucous cells and eosinophilic granular cells.

A qualitative assessment of the observations from both trial 1 and trial 2 was provided.

5.3 RESULTS

5.3.1 WATER QUALITY

The water quality parameters measured during the experimental trials 1 and 2 are tabulated in Table 5.1 and 5.2 respectively. Throughout the experimental period the ammonia concentrations in ammonia treated tanks remained almost constant. Although slight increases were noticed in the total ammonia concentrations of the control tanks over the experimental period, the mean values of ammonia did not differ vary much between the two replicate control tanks. The calculated unionized ammonia values for control tanks remained relatively low and well below the recommended "safe" levels of 0.016 mgl^{-1} UIA of U.S. Environmental Protection Agency (1976) and 0.021 mgl^{-1} UIA recommended by EIFAC (1970).

Table 5.1 Physico-chemical characteristics of the test solutions measured during the trout experimental period (trial 1) mean ± SE, n = 2

	NH_4Cl mgl^{-1}	TA-N mgl^{-1} Mean ± SE	NH_3-N* mgl^{-1} Mean ± SE	Temperature** ^oC	pH Mean ± SE	Dissolved oxygen mgl^{-1} Mean ± SE	Total Alkalinity $meql^{-1}$ Mean ± SE	Electrolyte Conductivity $nS\ cm^{-1}$ Mean ± SE	Nitrite-N (NO_2-N) mgl^{-1} Mean ± SE	Total Hardness mgl^{-1} Mean ± SE
Control										
	0.00	0.957 ± 0.023	0.009	15.0	7.56 ± 0.014	9.05 ± 0.00	8.45 ± 0.028	1655 ± 7.07	0.033 ± 0.024	24.67 ± 0.94
1	89.26	24.375 ± 0.177	0.234	15.0	7.55 ± 0.035	8.03 ± 0.172	8.39 ± 0.168	1845 ± 7.07	0.004 ± 0.001	24.00 ± 1.41
2	124.93	33.963 ± 0.185	0.311	15.0	7.53 ± 0.007	9.95 ± 0.071	8.43 ± 0.021	1930 ± 0.00	0.003 ± 0.0002	25.00 ± 0.95

*NH_3-N concentrations were calculated from the measured mean values of total ammonia, pH and temperature.

**Temperature was maintained constant at 15.0^oC.

Table 5.2 Physico-chemical characteristics of the test solutions measured during the trout experimental period (trial 2) Mean ± SE, n = 2

	NH_4Cl mgl^{-1}	TA-N mgl^{-1} Mean ± SE	NH_3-N* mgl^{-1}	Temperature** ^oC	pH Mean ± SE	Dissolved oxygen mgl^{-1} Mean ± SE	Total Alkalinity $meql^{-1}$ Mean ± SE	Electrolyte Conductivity $nS\ cm^{-1}$	Nitrite-N (NO_2-N) mgl^{-1} Mean ± SE	Total Hardness mgl^{-1} Mean ± SE
Control										
1	0.00	0.623 ± 0.001	0.009	15.0	7.75 ± 0.01	9.20 ± 0.04	9.17 ± 0.093	1650	0.002 ± 0.0004	29.25 ± 0.75
2	108.16	29.79 ± 0.051	0.430	15.0	7.73 ± 0.01	9.01 ± 0.16	8.81 ± 0.025	1900	0.001 ± 0.0003	26.75 ± 0.25
3	139.06	37.72 ± 0.059	0.57	15.0	7.75 ± 0.00	9.32 ± 0.15	8.75 ± 0.02	1950	0.002 ± 0.0003	29.3 ± 0.8

*NH_3-N concentrations were calculated from the measured mean values of total ammonia, pH and temperature.

**Temperature was maintained constant at 15.0^oC.

5.3.2 MORTALITIES AND BEHAVIOURAL OBSERVATIONS

No fish mortalities occurred in either of the two ammonia concentrations tested in trial 1. Initial hyperactivity and hyperexcitability were noted during the first 2 days, while on later days fish were more normal in their behaviour. Neither abnormal swimming nor neurological signs were characteristic at these levels of ammonia.

However, in trial 2 severe neurological signs were noted at both concentrations of ammonia tested (Plate 38). Frequent erratic and burst swimming patterns with occasional spinning and spiralling was more common at 0.57 mgl^{-1} UIA than at 0.43 mgl^{-1} UIA. Three fish from one of the replicate tanks at 0.57 mgl^{-1} UIA died between 48-96 hours, whereas two fish totally lost their balance and settled to the bottom showing only twitching movements and occasional spinning or spiralling movements.

In the second replicate tank at 0.57 mgl^{-1} UIA, two fish were found dead on day 2, whereas one fish remained on the tank, bottom paralysed and unable to swim.

These fish with severe neurological signs remained alive and active at the bottom of the tank most of the time laying flat or curved for the rest of the experimental period.

In general, rainbow trout were more sensitive and reacted very vigorously with more severe neurological signs than common carp at the highest levels of ammonia tested.

During the initial 24 hours of exposure the fish ventilated faster and more actively, which coincided with their severe splashing movements, but their opercular movements seemed to normalize later. However, in fish exhibiting severe neurological signs the breathing frequency was very much slower with frequent coughing activity.

5.3.3 HISTOPATHOLOGY

Gills from the control fish subjected to only buffered dilution water did not show any recognisable histopathological changes under light microscopic observations. The filament and the lamellar layout was normal (Plate 31). No hypertrophied chloride cells could be seen on the lamellae although occasional chloride cells could be seen in the interlamellar region. The epithelium covering the lamellae consisted of flatter and thinner squamous type epithelial cells with no apparent sign of swelling. The non differentiated cells seen on the sides of pillar cells appeared normal.

The mucous cells in the control fish contained normal structural features (Plate 34). However, their density on the filaments, gill raker and gill arch epithelium was quite variable. Some areas contained large numbers of multiple rows of mucous cells, whereas in some other regions they were found in a single layer with other immature and maturing cells lying within the basal and mid epidermal regions in the case of gill arch epithelium. Mucous cells were generally absent on the lamellae, although occasional cells of very small size could be visualized in histological sections stained with either alcian blue or PAS stains or a combination of both.

Eosinophilic granular cells were present infrequently in the control trout gills, but were not as dense as in the control carp gills. Their presence was particularly seen around the gill filament cartilaginous rod, especially at the base and at the tip of the filament. Smaller and elongated EGCs were seen sparsely spread along the length of the cartilaginous bar amongst the collagenous and the fibroblast cells. Their presence in the filament epithelium proper was rare. Similarly they were found in very small numbers in the gill septal and connective tissue regions (Plate 37a).

Apart from these normal structural components no alterations in the cellular structure could be seen in the control fish gills.

HISTOCHEMISTRY OF MUCOUS CELLS AND EGCs

The eosinophilic granular cells of trout gills, although staining distinctly with the haematoxylin and eosin stain, did not take up the PAS stain, unlike the EGCs of carp gills. They were also negative for alcian blue stain at different pH levels.

The mucous cell staining characteristics were different in different regions of the gills. When stained with either PAS or AB (pH 2.5) alone all the mucous cells stained positive but with the combined AB pH 2.5 and PAS stain a majority of the mucous cells on the arch and gill raker epithelium stained more with AB and less with the PAS stain. The mucous cells on the filament epithelium contained more PAS positive material than the AB positive substances (staining blue-pink).However, when

the tissues were stained with AB,pH 1.0 and PAS in combination, the mucous cells continued to stain PAS positively, and only rarely was AB stain taken up by any of the cells (Plate 34).

HISTOPATHOLOGICAL OBSERVATIONS AT VARIOUS AMMONIA CONCENTRATIONS

(a) AT 0.23 mgl^{-1} UIA

When compared with control gills a moderate increase in the number of chloride cells was obvious on the lamellae and within interlamellar areas. A clear swelling of these chloride cells was noticeable in the histological sections, although some lamellae showed normal flat epithelial cells in some areas of the sections. However, no recognizable necrotic changes were seen in these chloride cells.

An increase in the number of EGCs was also notable at this ammonia concentration. Such an increase was noticeable all along the length of the cartilaginous gill bars of the filaments, in closer contact with the gill cartilage than with the filament epithelium.

Increased numbers of mucous cells were present on the gill epithelium in general, but due to the considerable variations in their density from one area to another, and from one fish to the other, a clear comparison was not possible. Profuse and copious mucous secretions were more clearly seen on the epithelium in gill samples taken on the third day than those taken on the seventh day.

No alteration in the mucous cells were seen at this low concentrations of ammonia. The mucus pockets or the globules of mucus within the mucous cells remained clear and intact in the epithelial layers, and a clearly viscous mucus was seen in actively secreting mucous cells. No flocculations or vacuolations could be seen in the mucous cells at this ammonia concentration.

Thus cellular alterations were absent at this of ammonia concentration.

(b) AT 0.31 mgl^{-1} UIA

An increased number of chloride cells was uniformly represented on most of the lamellae, although the necrotic changes in the chloride cell were rare at day 3 (Plate 32a). The incidences of the appearance of pyknotic and necrotic chloride cells increased in fish gills exposed up to day 7.

A characteristically notable change at this level of ammonia was the appearance of small vacuoles within the cells and among the cells of the gill epithelium, typically representing spongiosis as a result of inter and intra-cellular oedema. Such spongiosis and inter and intra-cellular oedema of the epithelium was found to be increasing between day 4 and day 7. Such lesions were easily recognisable in histological sections as clear spaces within the epithelium (Plate 35a). Such cellular vacuolation was present in the lamellar epithelium and areas of the arch epithelium.

A moderate to increased vacuolation of mucous cells was noticed in the day 3 and day 7 gill samples respectively, in both gill arch and filament epithelium. Such vacuolation of the mucous cells was not only seen in the superficial layer of the epithelium, but some times also in cells of the deeper layers, probably in their immature or maturing stages (Plate 35b). Clear mucous cell hypertrophy was also seen on the filament epithelium. The vacuolations of the mucous cells as noted above were due to the flocculation or clumping of the mucous vesicles within the mucous cells, resulting in empty spaces and smaller accumulation of mucus debris.

(c) AT 0.43 AND 0.57 mgl^{-1} UIA

The basic pathological changes seen at all higher levels of ammonia (ie. 0.43 and 0.57 mgl^{-1} UIA) were the same, but the extent of these changes were greater at the highest concentration of ammonia, and at a given concentration of ammonia these changes were seen to be increased in the day eight samples. The characteristic changes were severe proliferation, hypertrophy and necrosis of chloride cells (Plate 32b,34a), increased cellular oedema and vacuolation of the epithelium (Plate 33b), mucous cell hypertrophy and vacuolation of mucous cells (Plate 36), and increased infiltrations of eosinophilic granular cells (37b,37c).

Apart from these above mentioned changes, extensive infiltration of cells, histologically noticeable as small cells with little cytoplasmic content and dark staining nuclei (resembling lymphocytes), was seen in the epithelium and interlamellar areas.

Plate 31.

A photomicrograph of the control trout gills after one week in the phosphate buffered dilution water.

(a) Perfectly normal control gills with no apparent effect of the phosphate buffer(H&E, 600X).

(b) Photomicrograph of the control trout lamellae showing normal lamellae with a thin layer of lamellar epithelium. (H&E,1500X)

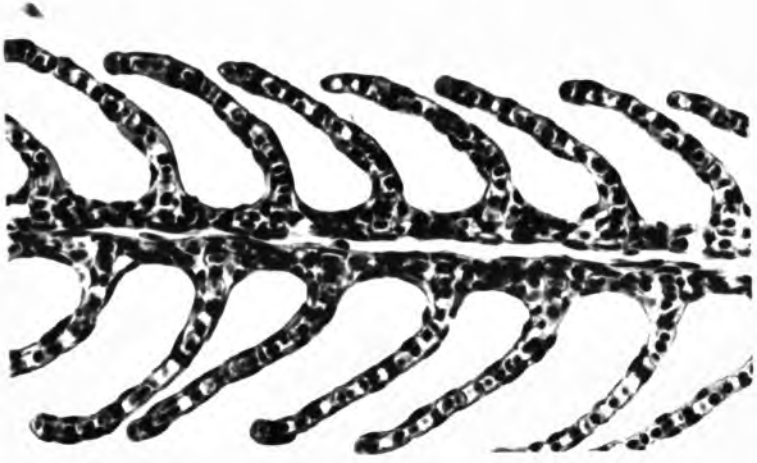


Plate 32.

(a) A photomicrograph of rainbow trout lamellae showing severe chloride cell proliferation as seen after 7 days exposure to 0.31 and 0.43 mgl^{-1} UIA concentrations. 1500X

(b) A photomicrograph of the lamellae from trout gills exposed to 0.43 mgl^{-1} UIA after 7 days experimental period. Note the necrosis of chloride cell (arrowed) (H&E, 1500X)

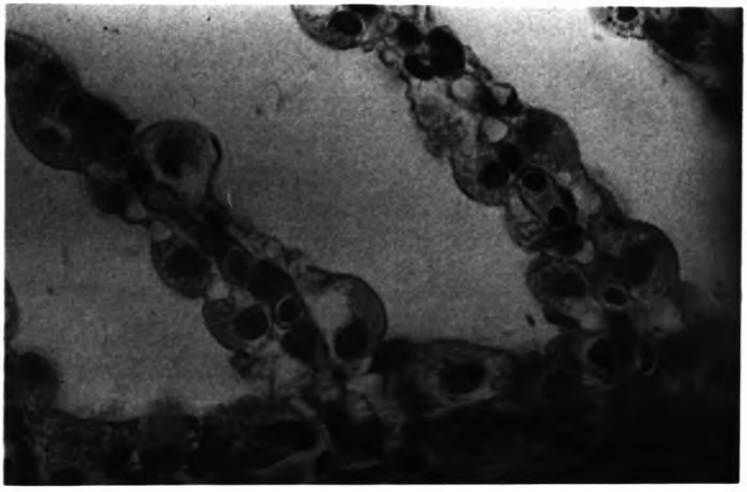


Plate 33.

(a) A photomicrograph of the lamellae of trout exposed to 0.57 mgl^{-1} UIA for seven days. Note the necrotic changes in the lamellar chloride cells (arrowed). 1,500X

(b) Severe epithelial vacuolations in the lamellar and filamental epithelium (oedema) at 0.57 mgl^{-1} UIA after 7 days exposure. 1500X

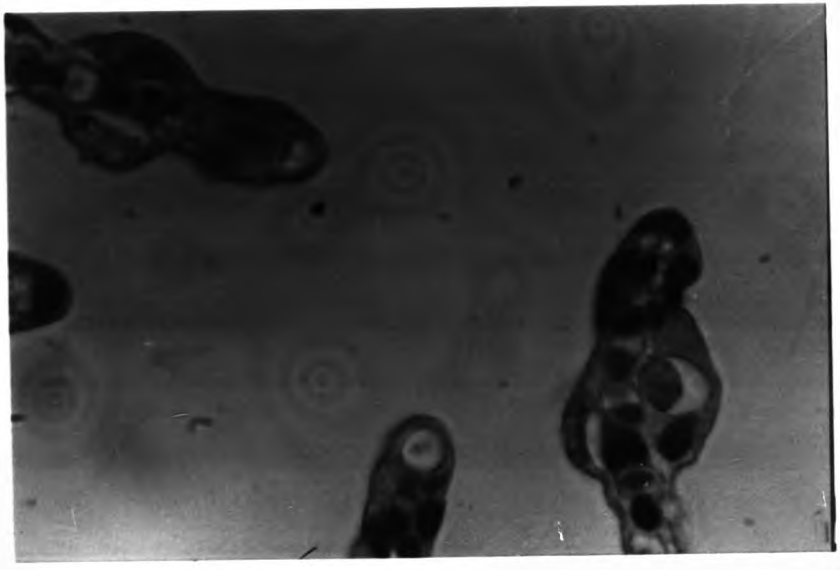


Plate 34.

A photomicrograph of the gill arch epithelial mucous cells of control trout after 7 days of experimentation.

(a) Normal mucous cells staining blue-pink in combined Alcian blue and PAS staining at pH 2.5 (375X).

(b) A photomicrograph of the same section as above stained with combined Alcian blue and PAS at pH 1.0 showing the predominance of strongly PAS positive mucous cells indicating the predominance of sialated mucus (375X).

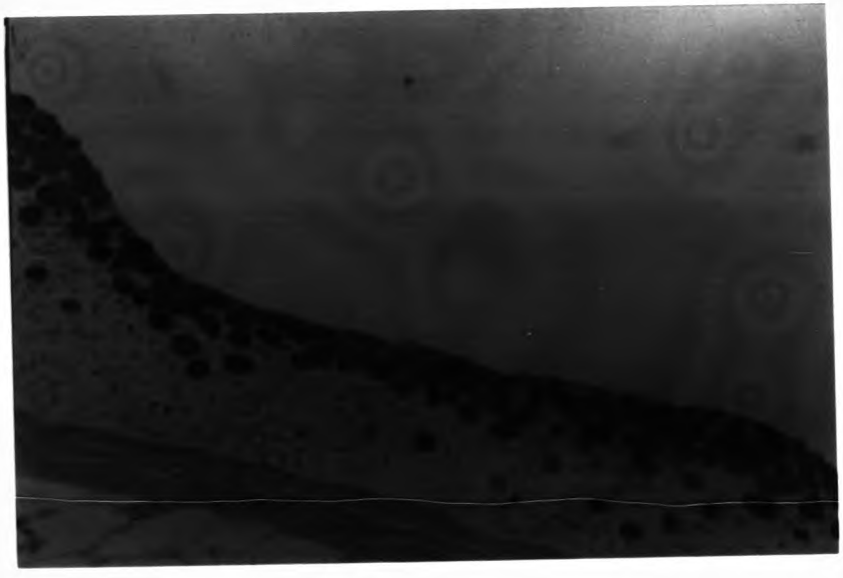
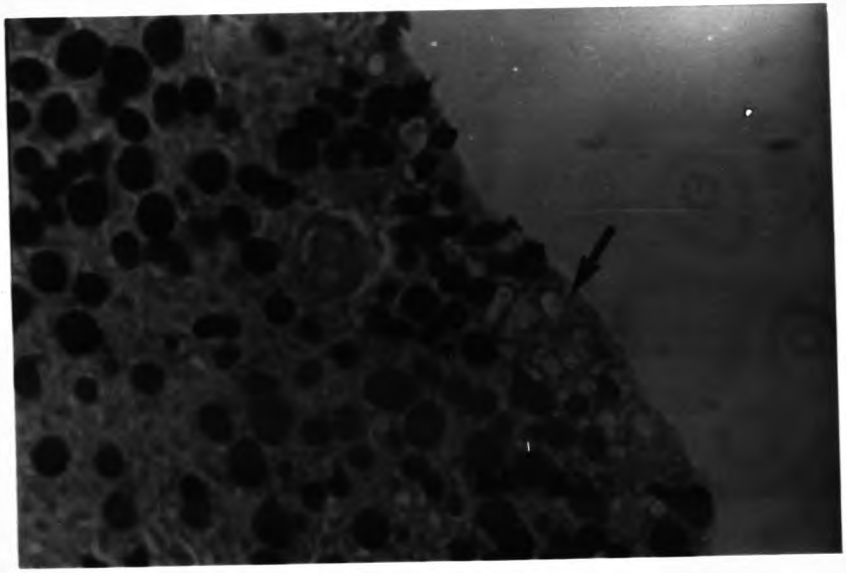


Plate 35.

(a) A photomicrograph of the trout gill epithelial mucous cells from fish exposed to 0.23 mgl^{-1} UIA for seven days. Note normal mucous cells and mild oedema of the epithelium (arrowed) (PAS, 600X).

(b) Gill epithelium of trout exposed to 0.4 mgl^{-1} UIA showing flocculated mucous cell contents and mild hypertrophy, also note mild oedema. 600X



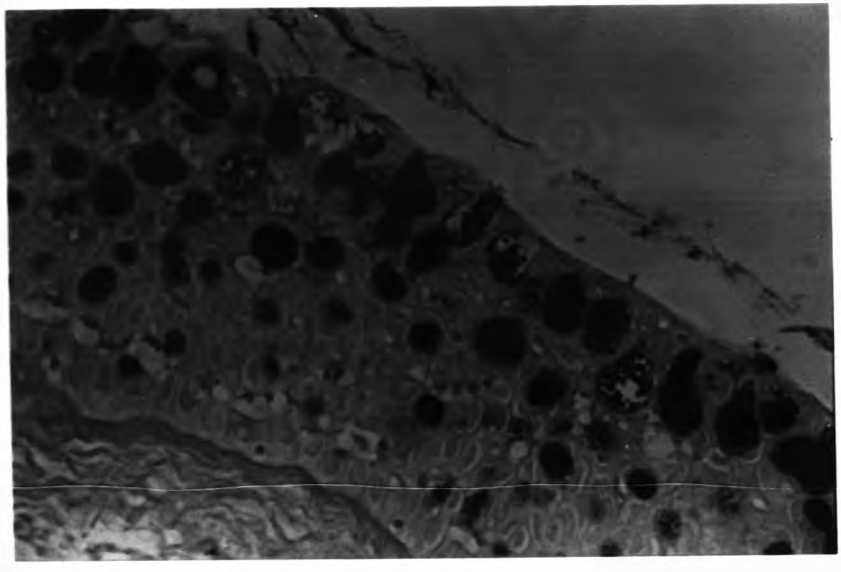


Plate 36.

(a) A photomicrograph of the trout gill epithelium exposed to 0.5 mgl^{-1} UIA for seven days showing severe mucous cell vacuolations and flocculation of mucus material in sections stained with combined AB/PAS at pH 2.5 (375X).

(b) Trout gill arch epithelium showing the cellular vacuolations and flocculation of mucus cell contents at 0.57 mgl^{-1} UIA for seven days, (No changes in the histochemistry of the mucous cells can be seen from those of the control treatments) (375X).

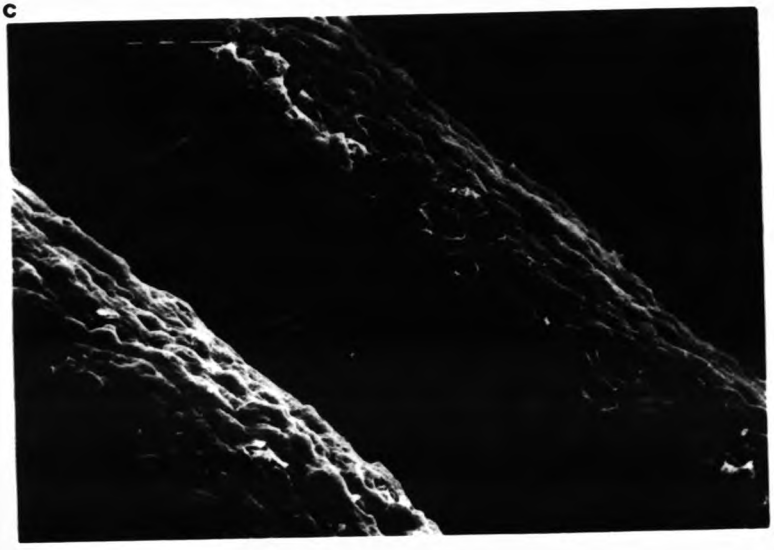
(c) A SEM of the trout gill filament, exposed to 0.43 mgl^{-1} UIA for seven days showing exhausted mucus cells on the filament epithelium (600X).

(d) SEM of the trout gill filament exposed to 0.57 mgl^{-1} for seven days. Note severe exhaustion and resulting damage to the gill epithelium (1200X).

a

b

c



d

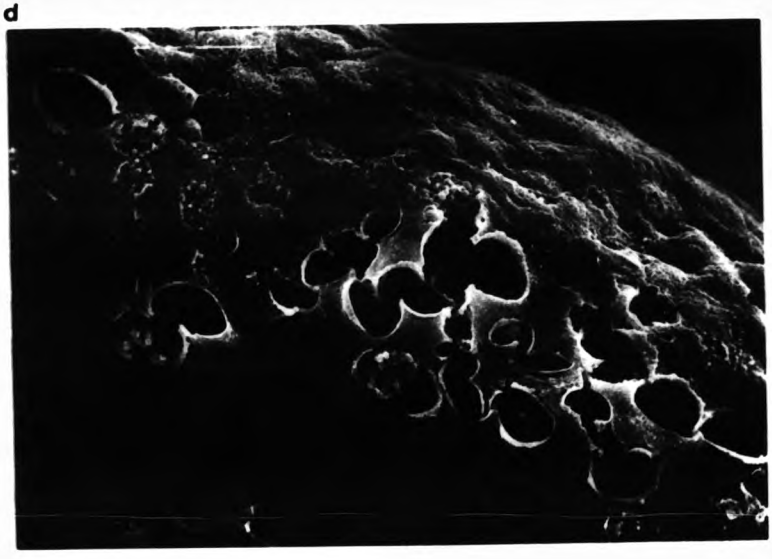


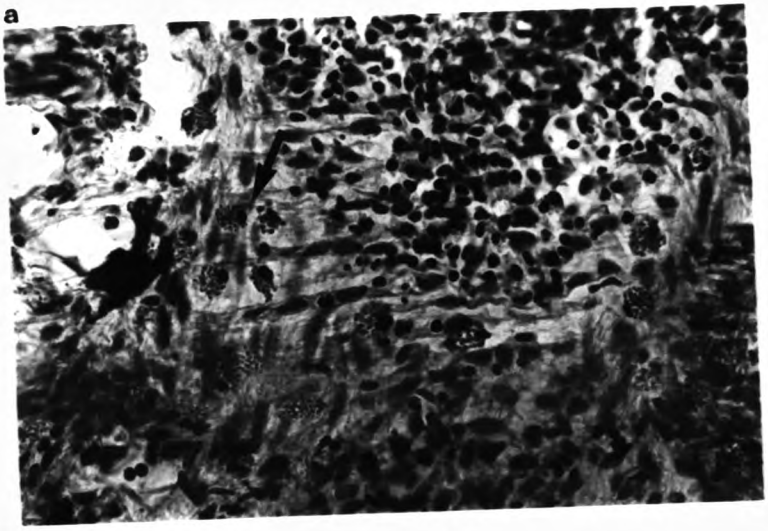
Plate 37.

(a) A photomicrograph of the control trout gills showing normal distribution of the eosinophilic granular cells in the connective tissues (arrowed) after seven days of experimental exposure.(H&E, 600X)

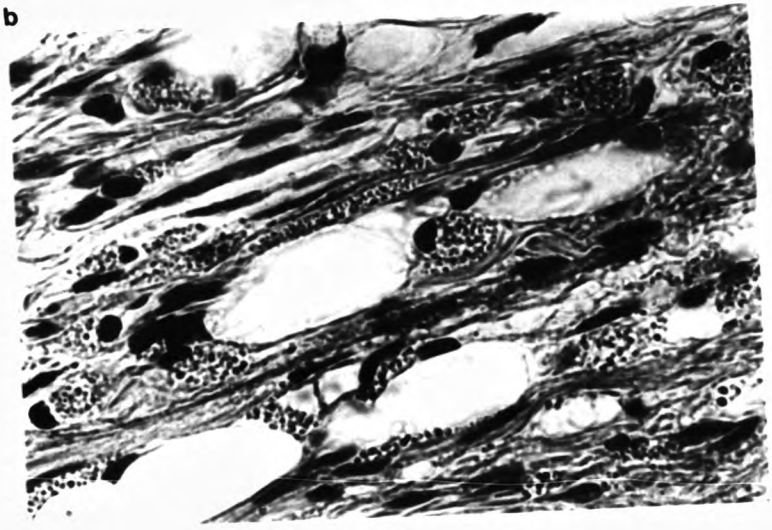
(b) Increased infiltration of the EGCs into the connective tissues after seven days exposure to 0.43 mgl^{-1} UIA. (H&E,1500)

(c) Severe infiltration of the EGCs into the gill connective tissues and their progressive degranulation and necrosis at 0.57mgl^{-1} UIA after seven days exposure. (H&E,1500X)

a



b



c

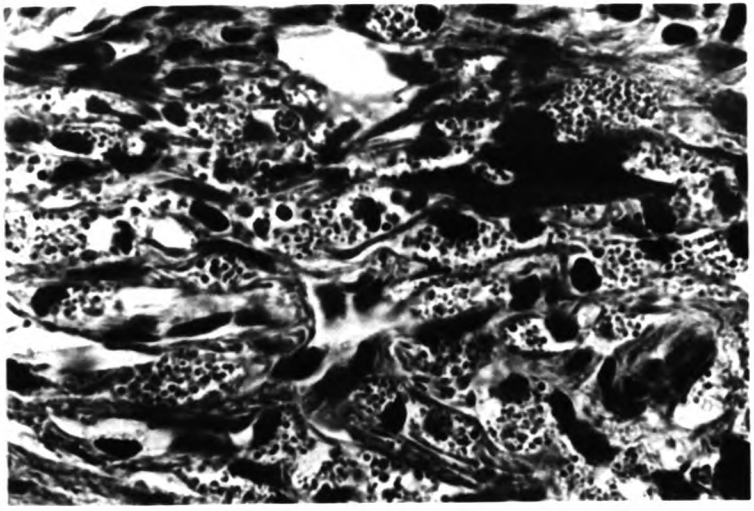


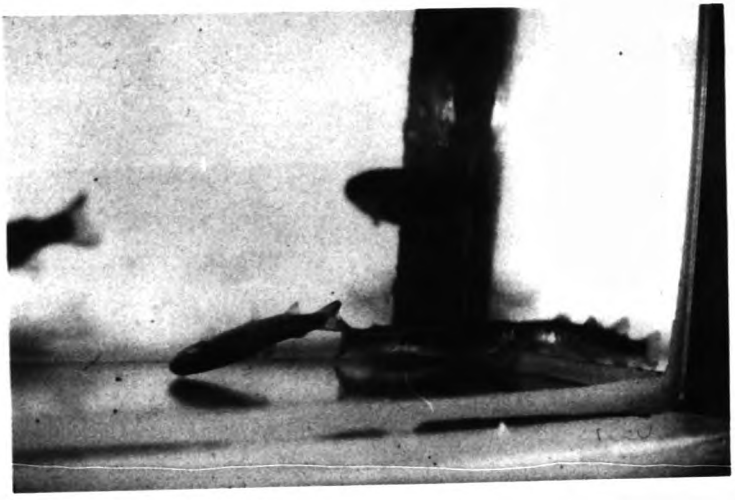
Plate 38.

Behavioural responses of trout in phosphate buffered experiment.

a) Control trout, note the normal behaviour of the fish in the experimental tanks.

b) Trout exposed to 0.43 mgl^{-1} UIA exhibiting loss of equilibrium and neurological signs.

c)Trout exposed to 0.57 mgl^{-1} UIA exhibiting loss of equilibrium and severe neurological signs.



Due to an overall increased cellular activity resulting from chloride, mucous and eosinophilic granule cells, the primary lamellae appeared relatively thicker than the control gills, although the lamellae were conspicuously thickened due to the proliferation and swelling of chloride cells on the lamellae.

At higher concentrations of ammonia *i.e.* 0.43 and 0.57 mgl^{-1} the EGCs were also found in large numbers in the connective tissues of gills and in the septal regions. Necrosis of EGCs was also noted in these tissues. Infiltration of EGC's into the primary (or filamental) epithelium was less, but their numbers increased within the vicinity of the cartilaginous gill bar,lying amongst loose fibroblast cells.

Although no epithelial damage could be visualized under light microscopy, scanning electron microscopy revealed the presence of severely distorted and widely open exhausted mucous cells in patches (or in groups) on most of the filamental epithelium (Plate 36c,d). Due to increased swelling of the mucous and chloride cells, the filamental epithelium was unevenly elevated, but no lesions such as those seen in the case of carp gills were noticed.

The micro-ridge pattern which was uniform both on the lamellar and filamental epithelium in the case of trout gills was unaltered.Filament tips were excessively thickened and showed severely distorted and increased mucous cell numbers.

Telangiectic lamellae were rarely seen, except for very low numbers on a few gill filaments, especially those of the short filaments on either extremity of the gill arches in whole mount

sections. Since their occurrence was only rare, and was sometimes also noted in a few of the controls, they were not thought to bear any relationship to the ammonia treatment. No rodlet cells were noticed in the trout gills, either in the normal control fish or in any of the ammonia exposed trout.

5.4.DISCUSSION

From the results of trial 1 and 2 in the present experiments on trout, it is very obvious that the pathological changes noted in trout were very much milder than those in the carp, and some changes that were seen in carp were totally absent in trout.

The greatest contrast was the absence of any effect of the buffered dilution water on the trout experimental controls as opposed to the severe alterations noted in control carp in experiment 4. The gill structure of trout controls in the present experiment as observed histologically was normal, displaying normal filamental and lamellar lay out (Plate 31) both after 3 and 7 days of exposure. Neither lamellar swelling, nor the characteristic infiltrations of different cell types, as noted in the carp experiments, were seen. Other tissue alterations noted in the experimental carp controls (Chapter 4.), such as excessive tissue hyperplasia and severe thickening of the filamental and lamellar epithelium, were absent.

These results indicated that under relatively identical water quality conditions as generally adopted in toxicological studies, the reaction of gills of different fish species gills were very different.

Although such reactions are difficult to interpret, these differential reactions to near identical water quality conditions by carp and trout may explain possible adaptational variabilities,and different biochemical and physiological mechanisms that enable them to react and adopt to varied environments in different ways.

It is also possible that trout, being a migratory fish capable of undergoing morphological and physiological adaptations for a life in fresh water and then sea water,like other salmonid fish, might also possess a genetically inbuilt capability for greater resistance to water containing high levels of dissolved solids, with appropriate physiological mechanisms and different subcellular organelles. On the other hand carp being a purely fresh water fish with limited capabilities to tolerate even slightly brackish water conditions might possess a different tissue and sub-cellular composition, being physiologically incapable of withstanding changes in water quality resulting from a high content of dissolved solids that might upset ionic balance.

Similarly, the histological changes that were noted at different ammonia exposure levels in identically buffered dilution water varied in that the trout manifested limited histological lesions at their respective acute and subacute ammonia concentrations, whereas the carp tissue reactions were more drastic and severe resulting in such lesions as nodular swelling of the gill epithelium with subsequent necrosis which was not present in trout.

These relative differences noted in the severity of ammonia caused histopathology seems to have also been reflected in the published literature. As indicated in the works of Flis (1968a, 1968b) the lesion types and the extent to which the gills were damaged seem to be of a far more severe nature than those of the histopathological descriptions provided for trout by Burrows (1964); Smith and Piper (1975); Smart (1976); Thurston *et al.* (1984) and Klontz *et al.* (1985).

The lesions observed in the present experiments on trout exposed to various ammonia concentrations are discussed below with respect to those specific lesions that have already been recorded for trout and other fish species.

The gill lesions that have been attributed to ammonia in the past include hyperplasia, hypertrophy and lamellar aneurysms (telangiectasis) which have been referred to by some as the characteristic lesions of ammonia exposure. Apart from these, lesions such as excess mucus secretion, oedema, necrosis of lamellar epithelium, capillary congestion, dilation of lamellar blood sinuses, infiltration of leucocytes, epithelio-capillary separation and cellular vacuolation are the other changes that have been recorded less frequently in the literature as a consequence of ammonia exposure. However, in the present study apart from excess mucus secretion, mucous cell necrosis, and vacuolation of the epithelium, the changes were less significant.

HYPERTROPHY

In the present study the most conspicuous pathological change that was associated with the trout gill epithelium exposed to different ammonia concentrations was that of lamellar chloride cell proliferation and hypertrophy leading to necrosis. Although such a change has not been specifically identified by earlier workers in ammonia toxicity studies as chloride cell proliferation, none the less it has been consistently identified and has been reported as hypertrophy of lamellar epithelium in trout (Smith and Piper, 1975; Burkhalter and Kaya, 1977; Thurston *et al.*, 1978; Thurston *et al.*, 1984; Klontz *et al.*, 1985). Similar descriptions were also given in studies on coarse fish (Redner and Stickney, 1979) such as Tilapia aurea under elevated ammonia levels and in the case of milk fish, Chanos chanos. subjected to acutely lethal ammonia concentrations for 72-96 hours (Cruz and Enriquez, 1982). The possible reasons why the above authors described this change only as that of epithelial hypertrophy could have been due to the following reasons:(a) Since, normally, chloride cells are restricted to the interlamellar epithelium and to the basal areas of the lamellae on the filament epithelium, it might have been a case of possible failure to reason as to why the chloride cells should appear on the respiratory lamellae of fresh water fish gills in response to ammonia. (b) Due to the fact that normal light microscopic histological observations do not easily allow the identification/characterization of chloride cells unless concurrent electron microscopical examinations are also conducted.(c) Thirdly, the later workers might have described been
interested in drawing their results in line with already

existing terminology.(4) Finally, chloride cells might not have been present in the sections examined by various workers, possibly due to their varied distribution patterns (for example some times they are only seen on the afferent filament sides of the lamellae) or due to the limited histological observations. This non-recognition of chloride cells by the earlier workers might also explain why no physiological explanation was provided for such a change noted that it (hypertrophy) resulted in the thickening of the lamellar epithelium thereby increasing the diffusion distance of respiratory gases and hence a reduction in the oxygen extraction efficiency and a possible reduced penetration of the toxicant. The osmotic or ion regulatory implications that could result from ammonia ($NH_3 + NH_4^+$) have not received attention in the past although the physiological consequences of high ammonia loading have been well documented in the recent literature (Armstrong *et al.* 1978; Cameron and Heisler, 1983;Tucker, 1985; Randall and Wright, 1987) (see chapter 4).

The gills of fresh water fishes serve a variety of functions of which the first and foremost is the exchange of respiratory gases, a function that necessitates a large and permeable surface area. As a consequence the gills are also the main site for the diffusional losses of ions (McDonald and Wood, 1981) that are compensated by active transport mechanisms which are primarily localized in the gill epithelium. Various exchange process such as the Na^+/H^+ or NH_4^+ exchange mechanism, Cl^-/HCo_3 or OH^- and the uptake of other ions such as potassium and calcium seem to take place through similar exchange processes as indicated above at the gills (McDonald, 1983), thus playing an important role in osmoregulation and acid-base balance.

Finally the gills are also the principal site of nitrogen waste excretion employing a combination of ionic and non-ionic diffusions and exchange against Na^+. Thus in this the gills assume much of the role that is the sole responsibility of the kidneys of terrestrial vertebrates. Hence it may be important to consider the functional complexity of fish gills with its structural delicacy and intimacy with the external environment in the interpretation of histopathological studies. Therefore, the effect of ammonia on gills may result in a variety of disturbances of which the chloride cell proliferation seems to be one of the most important initial responses. The processes that result in chloride cell proliferation and the need for the fish to eject ammonia from the body to survive, have been discussed in greater detail in chapter 4 on carp and the response of trout appear to be no different from that of carp with respect to chloride cell proliferation, hypertrophy and necrosis.

Chloride cell proliferation, hypertrophy, and/or necrosis have been reported under a variety of circumstances other than ammonia exposure. Table 5.3 lists such studies, clearly emphasing the sensitivity of the reaction and the significance of the problem of ionic and osmotic disturbance at the gill surface caused by various chemicals.

Although many questions relating to the role of chloride cells in each of the specific situations listed in Table 5.3 are not fully answered, the general concensus is that they are involved in either ion depletion or ion loss from the body fluids (such as exposure to deionized/distilled water, gill damage to parasites, skin wounds, acid stress), or in additional loading

of unwanted ions of various chemical pollutants of both heavy metal and inorganic origin (heavy metal salts of Cd, Pb, Zn, Co, Al and inorganic salts of ammonium and nitrite) that usually result in ion gradients due to their dissociation properties and build up in the physiological fluids of the fish body. This probably indicates a complex ion regulatory ability of chloride cells under situations of acute osmotic stress and thereby the maintenance of homeostasis of the milieu interior of the fish. However the necrotic changes noted in the present study, as well as those of others (Table 5.3) in which necrotic changes were recorded under different treatment conditions, may indicate the failure of chloride cells to survive under prolonged toxic conditions leading to biochemical and physiological degradation finally resulting in death.

Table 5.3 Agents other than ammonia that have been known to induce chloride cell proliferation, hypertrophy and/or their necrosis

Author(s)	Causative Agent	Fish Species	Results and Interpretations
1. Laurent and Dunel, 1980 Laurent, 1985	Deionized water Fungal disease Skin wounds	Rainbow trout	Lamellar Chloride cell proliferation (due to intense ion losses)
2. MacLay, 1985	Parasitic infection due to I. multiphilis	Rainbow trout	Chloride cell proliferation on the secondary lamellae (A defence mechanism against acute ion losses due to the break down of cellular integrity as a result of severe parasitic damage)
3. Matthiessen and Brafield, 1973	Exposure to dissolved zinc	Stickle Back Gasterosteus aculeatus L.	Proliferation of chloride cells and substantial thickening of the lanellar epitheliam (possibly to excrete zinc out of gill tissues).
4. Oronsaye and Brafield, 1984	Dissolved cadmium	Stickle Back Gasterosteus aculeatus	An increase in the number of chloride cells on the lamellae and their necrosis (possibly to eject cadmium and in response to an increased ionic regulation at the gill surface).
5. Crespo and Sala, 1986	Dissolved zinc ($ZnSO_4$)	Dogfish Scyliorhinus canicula	Chloride cell proliferation (a compensatory response to resolve altered osmoregulatory capacity)

Cont/...

Table 5.3 (Cont)

Author(s)	Causative Agent	Fish Species	Results and Interpretations
6. Karlsson-Norrgren et al. 1986a 1986b	Acid water and Aluminium exposure at low temperature	Brown trout *Salmo trutta*	Chloride cell proliferation on the lamellae and degeneration (a compensatory mechanism for increasing the ion uptake as more and more chloride cell degeneration due aluminium precipitation).
7. Evans *et al.* 1988	Aluminium and Acid	Rainbow Trout *Salmo gairdneri*	Chloride cell hypertrophy and proliferation on the lamellae at low pH and aluminium, and their consequent necrosis and decline in numbers (a compensatory mechanism in respose to ion deficiency and subsequent poisoning due to aluminium).
8. Leino *et al.* 1987	Experimental acidification of Lakes	Pearl Dace *Semotilus margarita* and Fathead Minnows *Pimephales promelas*	Proliferation of chloride cells (acid associated osmotic stress, i.e. abnormally high ion losses)

Cont/...

Table 5.3 (Cont)

Author(s)	Causative Agent	Fish Species	Results and Interpretations
9. Gaino, Arillo and Mensi, 1984	Exposure to Nitrite	Salmo gairdeneri	Accelerated chloride cell turnover, hyperactivity indicated by their hypertrophy and degeneration (to maintain cellular ionic stability with respect to chloride in particular, by involing ion exchange processes other than that of Cl-/HCO3 (such as Cl^{-1}/organic anion or Cl^{-1}/lactate) as nitrite intoxication inhibits the carbonic anhydrase activity.

HYPERPLASIA

Gill hyperplasia has been proposed as a common sign of chronic ammonia poisoning (Burrows, 1964; Larmoyeux and Piper,1973; Smith and Piper, 1975; Robinette, 1976; Cruz and Enriquez, 1982). In the present investigation on trout hyperplasia was not a characteristic lesion indicating that probably acute/sublethal levels of ammonia are not responsible for such a lesion. However, the similar absence of gill hyperplasia has been reported in chronic sublethal studies (Bullock, 1972; Smart, 1976; Redner and Stickney, 1979) wherein the fish were exposed to ammonia for over 30 days. Mitchell and Cech (1983) also failed to produce gill hyperplasia in adult channel catfish exposed to ammonia at 0.58 ± 0.18 mgl^{-1} UIA for 38 days, but when the fish were exposed to a combination of ammonia and the residual chlorine compound, monochloramine, severe hyperplasia resulted. Hence these authors concluded that use of public tap water containing low levels of chlorine (even typical of charcoal filtered public water) in ammonia toxicity studies could result in severe gill hyperplasia. Based on these results they speculated that the severe hyperplasia reported by Robinette (1976) in channel catfish was as a result of the combined effect of ammonia and residual chlorine (monochloramine) rather than due to ammonia alone, as Robinette (1976) used a charcoal filtered public water supply. Mitchell and Cech (1983) have also further suggested that the gill hyperplasia observed in salmonid studies (Burrows, 1964; Larmoyeux and Piper, 1973) designed to test a water reuse system could have resulted from elevated levels of bacteria and particulate matter rather than a direct effect of ammonia. However, recent studies by Soderberg *et al.* (1984), Thurston *et*

al. (1984), Klontz *et al.* (1985) and Soderberg *et al.* (1985) have further reported hyperplasia reconfirming the earlier results that ammonia results in gill hyperplasia.

Although these results lead one to think more sceptically, the possibility of prolonged chronic exposure to ammonia causing epithelial proliferations cannot be totally rejected. Elevated levels of ammonia are definitely capable of initiating a chloride cell proliferation, leading to their necrosis within a short period of time, and a prolonged chronic exposure may lead to such changes more slowly. When the chloride cells start necrosing, gill tissue may respond not only to augment the chloride cells but to increase the epithelial thickness due to the possible breach of osmotic balance as a consequence of gill damage (Laurent, 1984) and therefore hyperplasia of the epithelium may result.

Gill hyperplasia as a result of ammonia exposure was rejected by Daoust and Ferguson (1984) based on their experiments lasting for 84 days at 0.45 mgl^{-1} UIA on rainbow trout, and also by Mitchell and Cech (1983). However the fact that both of these authors declared that ammonia does not cause any gill pathology at all in their respective study species is contrary to previous author's results.

From the present observations it was noted that whether one is examining hyperplasia or hypertrophy, it may not be uniformly present all along the length of the lamellae or filaments. Hence unless large number of gill samples are sectioned at different depth planes and examined, limited histology may not always reveal such changes.

In cases where only smaller pieces of gills are sampled for histological sections such changes may be totally lost. For example Redner and Stickney (1979) reported histopathological changes in ammonia exposed tilapia gills based on observations from a single fish sampled from each treatment and such observations may not represent the true picture of histopathology.

In the present study at least a minimum of 4 gills from each individual fish were blocked together and sections taken at various depths of the whole mount of the gill tissues were examined. A minimum of ten fish were examined at each treatment level. The current observations did not reveal any hyperplasia of the epithelium due to ammonia in this study, although proliferation and hypertrophy of chloride cells was conspicuous.

TELANGIECTASIS

Telangiectasis did not form a recognisable and significant lesion in the present study. However, a few occasional lamellar dilations (telangiectic lamellae) were noticeable in a few fish gills,but such findings occured in both control and treatment fish.

Most fish gills were devoid of any lamellar dilations indicating that such lesions were not caused as a direct consequence of ammonia. When rare telangiectic lamellae were present, most often they were found on the short filaments on either ends of the gill arches in whole mount sections.

The presence of telangiectasis or gill lamellar dilations or aneurysms as referred to by various workers have however been reported by several workers as a consequence of ammonia exposure (Bullock, 1972; Larmoyeux and Piper, 1973; Smith and Piper, 1975; Cruz and Enriqnez, 1982; Thurston *et al.* 1984 and Soderberg *et al.* 1985). However, recent workers (Herman and Meade, 1985; Crespo *et al.* 1988) have indicated that such lesions are probably due to mechanical stressors such as killing the fish by a blow on the head (which some of the above mentioned authors adopted) rather than due to ammonia.

Smart (1976), in his ammonia toxicity studies lasting for 21 days on adult rainbow trout, in which the fish were killed by a blow on the head, reported telangiectasis as the most characteristic feature of chronic ammonia exposure based on the severity of such lesions. Hence it seems more probable that the lamellar aneurysms described by Smart (1976) were due to the method of killing rather than owing to ammonia. Although the methods of killing the fish has not been documented in studies of Bullock (1972), Larmoyeux and Piper(1973), Smith and Piper(1975), Cruz and Enriquez (1982), the method of killing the fish in case of Thurston *et al*, (1984) was again that of a blow on the head and hence the possibility of ammonia causing telangiectasis in this study is also doubtful.

According to Roberts (1978) telangiectasis is a characteristic pathological change of the gill associated with physical or chemical trauma and is commonly found in farmed fish after grading or pond transfer, or in association with parasitic conditions, but it may also occur in association with metabolic wastes or chemical pollutants.

However such lamellar telangiectasis has also been reported on a minor scale in many toxicity studies (Mallatt, 1985) involving various chemical pollutants and heavy metals in which the fish have been killed using appropriate anaesthetics. In view of these observations it has also been speculated (Herman and Meade 1985) that various chemical pollutants may reduce the cellular adhesion of pillar cells, so that with a sudden increase in blood pressure or as a result of violent thrashing (as when trying to avoid irritating anaesthetics such as quinaldine) the lamellar blood spaces may dilate or rupture resulting in telangiectasis.

In this study the fish were handled gently throughout the experimental processes and fish were then killed with an appropriate dose of benzocaine prepared in similar water quality as the fish were taken from, thus minimizing the sudden exposure of fish to water changes such as pH and temperature. This procedure might have been responsible for reduced trauma and hence the absence of any consistent significant number of telangiectic lamellae that could in any way be correlated to experimental ammonia concentrations.

MUCOUS CELLS

Mucous cells and the production of mucus, often in considerable amounts, is the most distinctive feature of teleost epidermis (Van Oosten 1957; Harris and Hunt 1975; Sibbing and Uribe 1985). The mucous cells are the second most commonly encountered cells, after the filament containing cells (Malpighian cells) in the epidermis (Harris and Hunt 1975).

However their distribution seems to vary in different regions of the epidermis (Pickering and Macey 1977).

In the present experiment, variations in the thickness of mucous cell-containing epithelium in gills belonging to both control groups as well as the treatment groups was evident.

Several hypotheses have been advanced to explain the function of mucous cells of the fish epidermis. These implicate mucus as being involved in various processes such as :

1. Prevention of colonization by parasites, fungi and bacteria.
2. Lubricating and locomotory functions.
3. Osmoregulatory functions.
4. Specific immunochemical protective roles (Pickering 1974; Pottinger *et al.* 1984).

Increased mucous cell activity or profuse mucous secretions have been noted under the influence of various chemical pollutants (Mallatt, 1985), parasites and handling (Pottinger *et al.*, 1984; Roubal *et al.*, 1987) and due to ammonia exposure (Flis,1968a; Smart, 1976; Smith and Piper, 1975).

The epidermis and mucus layer of a fish provide the first line of defence against infection by potential environmental pathogens and are of obvious survival value in the short term (Ingram, 1980). Increased mucus secretion under toxic conditions is known to bar the entry of toxicants into the fish when exposed to such chemical toxicants as copper and zinc (Kumar and Pant, 1981), indicating that an interaction

between the mucus and heavy metal ions takes place resulting in the formation of a film of coagulated mucus on the surface of gills, which interferes with the process of gaseous exchange.

Although no such interaction between ammonia and mucus has been reported, an excess mucus secretion has been implicated to interfere with or hinder gas exchange (Flis, 1968, Smart, 1975).

In the present experiments the early profuse mucus secretions (noted on day 4 samples) seen in the case of fish exposed to low level ammonia concentrations might represent an initial defence reaction, whereas the cellular changes noted at the later stage, wherein the mucous cells became not only exhausted but necrotic, were mostly as a consequence of continued ammonia exposure and cellular damage.

In the control fish, although the mucous cell density did not seem to vary drastically from day 3 to day 7 samples, an increase in the mucus production may have been due to an increased ionic concentration due to the presence of buffer. No necrotic changes could be seen in the control fish mucous cells (Plate 34).

In histology under combined Alcian blue and PAS staining, the mucous cells from the control fish showed discrete staining mucous cells, whereas in the case of high ammonia exposed fish in both 3rd and 7th day gill samples, and in low ammonia-exposed fish on the 7th day samples variable vacuolation was seen. These types of necrotic cells were either totally or

partially vacuolar containing none or very little mucus material and a total destruction of mucus vesicles of such mucous cells. Varying numbers of normal looking discretely staining mucous cells were also present in such fish.

The vacuolation and floculations of cellular mucus vesicles were noted not only in the bigger and sometimes greatly enlarged superficial (surface) mucous cells but also in the maturing sub epithelial mucous cells at various depths in the epithelium.

A possible interference of ammonia in the mucous cell metabolism seems to be evident in this study. A possible effect of buffer ions in addition to ammonia cannot at the same time be rejected. No histochemical changes in the mucus produced were noticed as a result of ammonia exposure. The staining properties of mucous cells remained the same as those seen in control fish and described in the earlier parts of the present experimental results.

Under scanning electron microscopy the superficial mucous cells of high ammonia exposed fish appeared as exhausted and vacuolated cells with very widely opened openings, quite distorted (Plate 36c,d) and resulting in severe damage to the epithelium.

Mucous cell vacuolation, exhaustion and sometimes their bulging over the surface and then being shed intact have been reported as a consequence of exposure to heavy metal pollutants (Kumar and Pant 1981) and parasitic infestations (Roubal *et al.* 1987).

In the present study it was also noted that the same mucous cell changes that were clear in AB and PAS stained sections were hardly recognisable in H&E stained sections. This might have been one of the reasons as to why no such descriptions have been described by other workers. Also it is possibly due to the fact that some investigations were more interested in reproduction of such changes as those described as characteristic ammonia lesions (eg. hyperplasia, hypertrophy and lamellar fusion: Daoust and Ferguson, 1984) while others' intentions might have been to verify only selected lesions, such as hyperplasia as in the case of Mitchell and Cech (1983).

Although the Department of the Environment (1972), Cruz and Enriquez (1982) and Thurston *et al.*(1984), have described epithelial vacuolation as a result of exposure of ammonia, whether such epithelial vacuolations included mucous cell vacuolations is unknown.

VACUOLATION OF THE GILL EPITHELIUM

Vacuolation of the epithelium, (both gill arch, filamental and lamellar epithelium) was another most important and conspicuous change noted in fish exposed to 0.31 mgl^{-1} UIA and above. An increase in this vacuolation of the epithelium was noted at the highest ammonia concentration tested (*i.e.* 0.57 mgl^{-1} UIA).

Thurston *et al.* (1984) noted extensive vacuolation in epidermis of rainbow trout exposed to chronic ammonia toxicity. They

suggested that it was presumably hydropic in nature and may have resulted from increased tissue permeability. Similar histological changes have also been reported in rudd, Scardinius erythrophthalmus, epidermis exposed to 0.08 and 0.16 mgl^{-1} UIA (Department of the Environment 1972). Similarly Cruz and Enriquez (1982) reported severe and extensive vacuolation in the lamellar epithelium of milk fish, Chanos chanos, exposed to 20.65 ppm total ammonia for 24 and 72 hours. This time period seems to be very much shorter than all the above mentioned studies but still resulted in extensive and more severe vacuolations. This may suggest a species specific difference once again or be due to other unknown water quality effects.

Such a tissue reaction, although resembling cellular spongiosis and inter and intra-cellular oedema, may be specifically due to an increased tissue permeability as stated by Thurston *et al.*(1984). Lloyd and Orr (1969), who measured the urine production of rainbow trout exposed to sub-lethal concentrations of ammonia, found a considerable increase in urine flow rates. The extent of this increase was directly related to the environmental unionized ammonia concentrations. Therefore Lloyd and Orr (1969) suggested that this diuresis reflects an increase in the permeability of the fish to water and thus postulated that death probably occurs when the increase in permeability exceeds the maximum sustained rate of urine production, although there was no direct evidence for this.

Speculating that "there is some justification for considering a disturbance of salt and water balance as a major toxic

action of ammonia" Smart (1975), in his own experimental investigations, found a significant increase in the body water content of trout exposed to ammonia concentrations above the lethal threshold limits but found no change in either plasma or muscle tissue water and electrolytes. These results suggest that tissues other than muscle were responsible for the increase (11.4% increase) in the body water content noted.

Thus the cellular oedema noted in the gill tissues exposed to increasing ammonia concentrations may well be a direct consequence of their increased permeability to water.

EOSINOPHILIC GRANULE CELLS

Eosinophilic granule cells (EGC's) were first described as such in 1971 by Roberts and co-workers as a distinctive cell type in the basal layers of the plaice (Pleuronectes platessa, L.) epidermis. They were identified as round ovoid cells with a marginally placed nucleus and packed with highly eosinophilic refractile granules within tissues fixed in formalin or dichromate-containing fixatives.

Similar cells have been reported by different earlier and later workers in various fish species and tissues such as in the sockeye salmon Oncorhynchus nerka (Bolton 1933), in the epidermis of gold fish Carassius auratus, (Percy 1970), in the oesophageal mucus of pike Esox lucius (Bucke 1971), in various connective tissues and epithelia of Atlantic salmon, Salmo salar and rainbow trout Salmo gairdneri (Smith 1975), in the epidermis of Ophisurus serpens (Anguilliformes) (Zaccone and Cascio 1979), and in the epidermis of red piranha, Serrasalmus

nattereri. (Zaccone 1980).

However, increased infiltrations of these EGCs into the epithelia and various other connective tissues under certain environmental and/ or disease conditions has caused scientists to speculate as to their importance and possible role in the functioning of the fish.

Incidences of increased infiltration of EGCs have been found and reported in the nostril mucosa of Atlantic salmon suffering from ulcerative dermal necrosis (Roberts, 1972), in the healing wounds of salmonid connective tissue (Roberts *et el.*,1974), in regenerating superficial skin wounds of Rita rita (Mittal and Munshi, 1974), in salmonid fish subjected to prolonged (up to 50 days) starvation (Smith, 1975), in the gill tissues of carp, Cyprinus carpio, exposed to chlorinated lime water and carp infected with parasitic trichodinids and Chilodonella (Hornich and Tomanek, 1983), and in the gill tissues of carp suffering from ammonia intoxication (Kovács-Gayer, 1984), in the lamina propria of the intestine of sergeant major fish Abudefduf saxatilis, suffering from microsporidian (Glugea sp) infections (Reimschuessel *et al.*, 1987). Therefore it is quite clear that, though EGCs are found in various tissues of different fish species, they are more often actively associated with infectious or non infectious disease conditions.

Although specific studies on the EGCs of different fish species to identify their functions are lacking, based on the available histochemical and circumstantial evidence it has been suggested that they play an active role in protecting

vulnerable areas of the fish from the damage of ionic, osmotic or pathogenic agents (Smith, 1975). The granule cells have also been credited with serving a number of functions such as a lipophase activity, the elaboration of antitoxins against pathogens, and a mast cell or globular leucocyte function (Al-Hussaini, 1949; Jordon and Spidel, 1924; Roberts *et al.*, 1972; Ellis, 1974).

The reaction of EGCs in response to ammonia in salmonid fish in general and trout gills in particular is unknown, although a characteristically increased infiltration of EGCs into the gill tissues and their necrosis has been recorded as a response to ammonia exposure in carp (Kovács-Gayer 1984; and in the present study chapter 4). It is possible that it may constitute a part of the inflammatory response of the gill tissues and other mucosal tissues.

According to Roberts (1972), in the salmonids an eosinophilic granule cell is occasionally found in the epidermis, but it is not a constant feature and is more frequently seen under pathological conditions. Although the earlier part of this statement may hold true for epidermis (skin), the gill epithelium and connective tissues of healthy trout seem to contain an identifiable and regular but low number of EGCs (unlike the normal carp gills in which the EGC populations are relatively higher). These are mostly seen in the basal regions of the filament epithelium along the length of the cartilage in the close vicinity of the cartilaginous cells and other connective tissues.

However, their increased numbers in the gill epithelium and connective tissues of fish exposed to different ammonia concentrations indicats a possible direct relationship between the EGCs and toxic ammonia concentrations. Although this observation further agrees with the suggestion of Al-Hussaini (1949) that the granule cells might have an antitoxic effect, the exact mechanisms involved in such an effect are unknown.

It was noticed in the carp gills that the EGCs of high ammonia treated carp underwent severe vacuolation of the granules (degranulation), indicating a probable release of the contents of the granules, whereas the granules in the control fish gill EGCs remained almost intact, which otherwise suggests that the EGCs might perform an effector function in the alleviation of the toxic effect of ammonia. This may also be the case in rainbow trout as an increased infiltration of the EGCs was clearly evident in ammonia exposed trout as opposed to control trout gills. Although it is reasonable to assume that in the present experiments (both on carp and trout) the increased infiltration of EGCs were noted as a direct response to ammonia exposure and hence have a probable role to alleviate the toxic effect, two of the changes noted here are conspicuously different for carp and trout. Firstly, the staining properties of the EGCs are different in carp and trout. Secondly, the extent of the EGC infiltration and necrosis is much greater in ammonia exposed carp than in ammonia exposed trout, suggesting species variation in populations of EGCs.

Extensive histochemistry carried out by Roberts *et al.* (1971), on the EGCs of Pleuronectes platessa, failed to enable them to place these cells in any of the usual cell classes of the epidermis, at the same time recognising them as different from blood eosinophil cells of teleost fish by being not able to stain by the carbol chromotrope method which is considered specific for eosinophilic cells. In the previous experiments (chapter 4) the EGCs of carp stained strongly positive for PAS indicating a predominance of neutral mucopolysaccharide and glycoprotein content of the EGC granule, while staining negatively for Alcian blue (AB) at pH 2.5 and 1.0 which is an indication of the absence of any traces of acid mucopolysaccharides. On the other hand the EGCs of trout did not take up either PAS stain or Alcian blue at pH 2.5 and pH 1.0, indicating the absence of both neutral and acidic mucopolysaccharides and mucoproteins. Similar observations have been noted in salmonid fish in which the EGCs stained negatively for PAS, at the same time failing to show any metachromasia with toluidine blue staining (Smith,1975; Vallejo, 1987) in wax embedded formalin fixed tissues. However a positive reaction for lipids and protein has been noted in the case of trout EGCs (Smith, 1975).

Further confusing variations have also been recorded in that in some fish the EGCs stained for both PAS and AB stains (Zaccone,1980) and in some others they did not stain for either PAS or AB (Zaccone and Cascio,1979).

In the present study it was also noticed that the EGC granules of both carp and trout stained positive for toluidine blue in resin sections processed for electron microscopy, an

observation which has also been reported by Vallejo (1987)in the case of trout implicating a probable loss of acid mucopolysaccharide substances from the EGC granules in the conventional formalin/dichromate fixation procedures. Therefore, although it appears that the eosinophilic cells described by various authors in different fish species seem to conform to general characteristics such as (a) their strong affinity to H&E stain (eosinophilic),(b) their presence in the epithelia,(c) their increased infiltration into the epithelia and connective tissues under conditions of disease or physical or physiological insult, their tinctorial properties, which may indicate their chemical composition and thus assist in the interpretation of their functions and probable origins, remain variable and often confusing.

Apart from these discrepancies in the tinctorial nature of EGCs, their definitive identification in the fish tissues has been further confused because of the occurrence of various other cells besides the characteristic EGCs of Roberts *et al.* (1971), such as the acidophilic granular cells (AGCs) of Blackstock and Pickering (1980), the sacciform cells described by Mittal and Agarwal (1977) & Whitear and Mittal (1986) and the rodlet cells described by Mattey *et al.* (1979) and Karlsson (1983).

Although the cells described by Blackstock and Pickering (1980) were called acidophilic granular cells (= eosinophilic cells) they encountered a confusing tinctorial nature of these cells and in their later study Pickering and Fletcher (1987), claimed that these AGCs are a type of serosal mucous cell analogous to those described by Whitear (1986). Similarly the

EGCs described by Zaccone and Cascio (1979) and Zaccone (1980) seem to resemble such cells rather than the EGCs of Roberts *et al.* (1971), Smith (1975) or those noted in the present study in trout and carp. Further more, the rodlet cells which are also eosinophilic and have been known to occur in different organs of various fish species (Leino 1974; Mattey *et al.* 1979, Karlsson, 1983), could easily be mistaken for EGCs when these cells are cut while sectioning in the histological preparations.

Thus, presently, a comprehensive knowledge as to the occurrence or absence of these various cell types in the epithelia of different fish species is lacking. In addition ,due to the confusing tinctorial properties of the EGCs that have already been described by various workers, any speculations as to the function and origin of EGCs seems indeed very uncertain and hence it is inappropriate to generalize on their functions.

Possibility of EGCs contain lysozyme or contribute to extracelluar lysozyme activity of mucus has been suggested. Coincident with the observation that among the marine teleosts granule cells are particularly common in the Pleuronectidae (Roberts *et al.*, 1971), Fletcher and White (1973), found that among the teleosts caught in British waters the lysozyme was consistently present throughout the year in the sera of Pleuronectidae, whereas with others (haddock) it was variable and in some other fish (such as cod) it was never present in detectable amounts. Although they demonstrated that it was the leucocyte fraction and not the red cell fraction that contained the lysozyme (which is in agreement with mammalian

findings) present in the sera, they concluded that the origin of the secretory lysozyme (found in external cutaneous mucus) may be different.

Based on the knowledge that in the plaice skin it has been possible to demonstrate histochemically distinct lysozyme containing cells which are absent from cod epidermis,& which do not appear to be leucocytes and occur both in the basal layers of the epidermis and migrating towards the surface, Fletcher and White (1973) suggested the possibility that the eosinophilic granule cell whose function is unknown might be related to such lysozyme containing cells.

Such a possibility, however, needs to be further investigated in different fish species along with the speculated functions of EGCs in disease conditions, in order to understand the exact role played by EGCs in the fish epithelia in general.

In summary the major tissue lesions due to ammonia in trout gills constitute:
(1) a severe chloride cell proliferation and their progressive necrosis,
(2) an increased infiltration of the EGCs in to the gill epithelia and subsequent degranulation.
(3) excess mucus production, mucous cell exhaustion and flocculation of the mucus substances.
(4) and cellular necrosis characteristic of pyknosis and karyorrhexis.

However,tissue lesions such as telangiectasis, and hyperplasia did not constitute the characteristic lesions

either at near lethal or at sublethal concentrations of ammonia tested in the current experiments. The lesion type that has been described as epitheliocapillary separation (ECS) (Klontz, et al. 1985) was also absent.

The distinctive differences in the histopathology noted in trout from that of carp were (1) the absence of the characteristic nodular swellings on the gill epithelium (2) and the absence of the detrimental effect noted in the carp gills due to the phosphate buffer.

Apart from these distinctive differences the various pathological processes such as those seen in the chloride cells, EGCs,and the epithelial cells were less severe than in carp.

CHAPTER 6

EFFECTS OF AMMONIA ON THE GILL STRUCTURE OF TROUT AND CARP IN TRIS-BUFFERED DILUTION WATER

6.1. INTRODUCTION

The gross histological and pathological changes observed in experimental trials on carp (Chapter 4) and trout (Chapter 5) were conspicuous under light microscopy and were readily reproducible.

In these above experiments, since all the important water quality parameters were clearly known, the cause of lesions in the control carp, which were dissimilar to those found in the carp exposed to different ammonia concentrations, could not be attributed to anything but the phosphate buffer used in these experiments.

Buffers such as those used in the present experiment are extensively used in various experimental studies and have been recommended for use in experiments wherein the choice and maintenance of proper pH is critical, especially in tests conducted with readily ionizable compounds (Murthy,1986a).

Although the wisdom of such a recommendation became questionable, at least in the case of carp, the absence of any such changes in the control trout (trials 1 and 2 of Chapter 5) subjected to similar buffer concentrations raised interesting contrasts and cast doubts on the continuation of the use of this buffer in these comparative studies in which the buffer used exerted a differential effect on the two different experimental fish species. In spite of the fact that the lesions noted in carp and trout exposed to increasing ammonia concentrations could be histologically correlated with

severe gill lesions present in high ammonia exposed carp could not be taken unequivocally as lesions caused by ammonia alone for the following reasons: firstly, the phosphate buffer itself brought about considerable gill damage in the control carp, and secondly, the type of nodular lesions seen in high ammonia exposed carp were not seen in trout, and have not been reported in carp before.

Hence a second set of experiments had to be planned (comprising basically the repetition of the earlier trials) to verify the nature of the occurrence of the histological and histopathological changes seen in the phosphate buffered ammonia toxicity tests by utilizing an alternative buffer to stabilize the pH.

At this stage however, a survey of the available literature wherein buffers have been employed particularly in ammonia toxicity studies, was conducted and it was noticed that such studies have employed a range of buffer chemicals either singly or in combination with various other chemicals.

Table 6.5. gives a typical example of the way in which these buffers have been used. Unfortunately most of these studies were not histological investigations and few of those in which histology has been carried out have either identified or reported the effects of any of the buffers used.

However, in one of the early investigations (McFarland & Norris 1958), studying the efficiency of buffers in live fish transportation in comparison with previous works wherein different inorganic chemicals (inorganic phosphate salts) had

been employed in fish seed transportation , it was noted that conflicting results were reported in which some authors had claimed beneficial effects (Vaas, 1952; Srinivasan, Chacko and Valson, 1955), while others (Saha *et al.*, 1956; Nemoto, 1957) had noted detrimental effects of buffer usage. Therefore, based on their own experimental evidence in different species of both fresh water and marine fish subjected to prolonged fish transportation, McFarland & Norris (1958) recommended that the organic tris-buffers they experimented with were most efficient in stabilizing acidity levels both for marine and fresh water application and that they had no detrimental effects on any of the 29 species of fish tested. Accordingly, in recent years the Sigma Chemical Company (SIGMA) has been providing tris-buffers not only for biochemical, enzymological and tissue culture studies but also for fish transportation and maintenance for prolonged periods of time (up to one week).

In the second set of experimental procedures reported here tris-buffer was adopted in place of phosphate buffer based on an initial trial experiment that promised the stability of pH.

Although tris-buffer has been used by Rice & Stokes (1975), in ammonia toxicity studies on rainbow trout at a concentration of 0.05 M, in the present experiments a lower concentration was adopted as for phosphate buffer (0.01 M), due to the large quantities of these buffer chemicals required at higher molar concentrations.

An initial trial was carried out to assess the stability of the pH, with tris-buffer of 0.01 M concentration in 50 ppm hardness dilution water and indicated that the pH was relatively stable

over a period of 3 days when 10 carp were used in 80 litres of water held in a glass tank. Also the histology of these fish exposed to tris-buffer for 3 full days did not show any deleterious effects on the gill tissues.

Hence it was decided to repeat the earlier experiments conducted on carp and trout, but using tris-buffer and thus verify whether similar pathology to that seen in high ammonia exposed carp in the phosphate buffered dilution water was reproducible. It was also decided to verify whether a prolonged exposure in tris-buffer (seven days) induced any changes in the control carp gills.

EXPERIMENTS ON TROUT

6.2.MATERIALS AND METHODS

Two experimental trials were carried out. In the first trial a dilution water of 30 ppm hardness was used with 0.01 M tris-buffer to stabilize the pH.

As the histopathology noted in the first trial was not any different or more severe than in the previous experiments (Chapter 4) a second trial was carried out in a dilution water of 250 ppm hardness while the concentration of the tris-buffer used remained the same (0.01 M), to determine whether the hardness altered the histopathology in any way, although a change in the hardness was not known to have any influence on the toxicity of ammonia (Thurston *et al.* 1975).

The rainbow trout used in the first trial weighed 15.92 ± 3.16 grams (±SD) and measured 11.77 ± 0.90 cms (±SD) while the fish used in the second trial had a mean weight of 9.72 ± 2.24 grams (±SD) and a mean length of 10.06 ± 0.97 cms (±SD).

The experimental system used and the other procedures followed were similar to that used in the experiments with trout described in Chapter 4.

Three concentrations of ammonia were tested in trial 1 and two concentrations in trial 2 with high hardness. In each of the trials the treatments were carried out in replicate tanks along with a duplicate set of control tanks.

Prior to experimentation the fish were acclimated to their respective dilution waters for 4 days, and to the buffered dilution water for 2 days, and were transferred to the experimental tanks as necessary.

6.3. RESULTS

6.3.1 WATER QUALITY

The mean values of pH and temperature measured throughout the experimental period, along with the other measured water quality parameters, are tabulated in Tables 6.1 and 6.2. The mean nitrite concentrations measured for the experimental period in both trial 1 and trial 2 did not exceed the safe limits recommended for salmonoid fish (EIFAC 1984).

However, one of the most interesting and peculiar aspects noted in these experiments was that of the interference of tris-buffer with the measurement of ammonia. The ammonia levels that could be measured were disproportionately variable and hence the actual estimations of ammonia could not be made during the experimental period. When the water samples from high ammonia containing treatment tanks were appropriately diluted with distilled water (as in the previous experiments), for the measurement of ammonia, the absorbance obtained was once again disproportionately low, resulting in low values of ammonia, but when the undiluted water samples were used with the same chemicals there was no blue colour development at all and the resulting absorbance fell below that of the reagent blank. This was also the case for water samples from the control tanks where no ammonia had been added.

Although some values could be obtained when the water samples were diluted, as these values did not represent the actual ammonia levels expected, they were not utilized.

Since the exact amounts of ammonium chloride added per litre of water in the case of ammonia treatment tanks was known, the nominal values of total ammonia expected have been provided. Therefore, the unionized ammonia concentration calculated based on these nominal total ammonia values at the measured mean temperature and pH values were considered as the actual experimental unionized ammonia values and are provided in the tables.

However, to indicate the nature of the interference of the tris-buffer in the measurement of ammonia, the actual measurable values of ammonia are also tabulated alongside the nominal values. Plate no.53 clearly indicates the colour development pattern of the tris-buffered ammonia containing water.

6.3.2 MORTALITIES AND BEHAVIOUR OF FISH

6.3.2.1 TRIAL 1 (Low hardness experiments)

The control fish remained normal throughout the experimental period. However, the fish exposed to the lowest ammonia concentration of 0.26 mgl^{-1} UIA appeared distressed. Such distressed behaviour was noted after 24 hours of exposure. The fish became dispersed all over the tank and occasionally exhibiting erratic swimming behaviour. But no severe neurological signs or mortalities were noted.

Amongst the fish exposed to 0.38 mgl^{-1} UIA signs of distress were noticeable within a few hours of exposure, and progressively the fish developed mild to variable neurological signs. After 72 hours of exposure 3 fish became severely affected. Of these 3, one fish floated with its belly up probably as a consequence of frequent surfacing and gulping of air, while the other two slowly sank to the bottom where they lay flat, partially paralysed with gill ventilation being slowed down. Such fish showed occasional erratic movement but no mortalities occurred throughout the experimental period.

At 0.49 mgl^{-1} UIA, 2 fish died within seven hours of exposure and by about 24 to 48 hours most fish became severely distressed and developed neurological signs. Within this period of time 2 fish floated with distended abdomens while 3 fish sank to the bottom and lay flat. The number of fish that floated increased from day 2 onwards while 3 fish that sank to the bottom remained there. On the other hand one or 2 fish remained relatively less affected. No further mortalities were noted after the initial death of 2 fish, inspite of severe neurological signs noted.

6.3.2.2.TRIAL 2 (HIGH HARDNESS EXPERIMENTS)

The fish in the control tanks appeared unaffected and normal. The fish exposed to 0.29 mgl^{-1} UIA, although showing relatively minor behaviourial changes initially, by day 5 most of the remaining fish in the tanks showed neurological signs as noted by their abnormal swimming on their sides due to the loss of equilibrium and occasional erratic swimming.

The fish exposed to 0.40 mgl^{-1} UIA were more severely affected. The fish started to lose their normal upright, swimming position within 10 hours of exposure. Such fish began to roll on their sides as they swam but attempted to regain their normal position. By about 20-24 hours a few of the smaller fish turned upside down and by about 48 hours most fish were severely affected and exhibited typically severe signs. One fish died on between 48 and 72 hours. Although 50% of the fish from each tank had been sampled at the end of day 3, further mortalities were encountered amongst remaining fish on day 4 resulting in the death of 3 more fish. At this stage, since most of the remaining fish exhibited grossly compromised, all the fish in this particular treatment were sacrificed at the end of day 4 for histological examination.

The fish in other treatments (ie 0.29 mgl^{-1} UIA and the controls) were held in the tanks continuously until the end of the stipulated experimental period of 7 days before they were sampled.

HSTOPATHOLOGICAL CHANGES

6.3.3.1.TRIAL-1, LOW HARDNESS EXPERIMENT

Histopathological changes noted in the gills of trout exposed to ammonia concentrations of 0.26, 0.38 and 0.49 mgl^{-1} UIA in tris-buffered dilution water did not differ very much from the changes that were already noted in experiments conducted with phosphate buffer.

The control trout gills remained normal and did not show any changes in the structure of lamellae or the filaments (Plate

39), although histologically the filamental and arch epithelia were found to be more richly endowed with mucous cells than those of the ammonia treated gills in sections stained for mucous cells. But no mucous cell necrosis or cellular oedema could be noted in the control gills. The lamellar epithelium remained flat and devoid of any chloride cells.

In fish exposed to various concentrations of ammonia increased chloride cell proliferation was characteristic. At higher concentrations of ammonia such chloride cell swelling occurred indicating the hypertrophic reaction of these cells. The inter and intra cellular oedema (histochemically recognizable as generalized vacuolation) was increased with increasing concentration of ammonia.

The severity of the chloride cell proliferation on the lamellae and the proliferation and necrosis of the mucous cells on the filament epithelium was clearly noticeable on the gill surfaces of the fish exposed to higher concentrations of ammonia (Plates 40,41 and 42b,c). Although the presence of large numbers of exhausted mucous cells on the filament epithelium in CYME studies indicated an increased mucous cell activity, the nonstainability of these cells in histological sections and their vacuolation, particularly at the surface epithelia was possibly due to the exhaustion of mature stainable mucous cells and/or necrosis of immature or maturing cells rather than an actual reduction in cell production.

Table 6.1 Concentrations of ammonium chloride and ammonia used in the test solutions and other measured physico chemical characteristics (trial 1 - trout experiment) mean ± SE, n=2

	Temperature °C	pH Mean ± SE	Dissolved Oxygen mgl^{-1} Mean ± SE	Total Alkalinity $meq\ l^{-1}$ Mean ± SE	Electrolyte Conductivity /nS cm^{-1} Mean ± SE	Nitrite-N NO_2-N mgl^{-1} Mean ± SE
Control	15.0	7.64 ± 0.01	8.89 ± 0.39	1.99 ± 0.03	1000 ± 0.0	0.012 ± 0.001
1	15.0	7.62 ± 0.14	9.08 ± 0.09	1.78 ± 0.09	1200 ± 0.00	0.003 ± 0.001
2	15.0	7.64 ± 0.01	9.25 ± 0.15	1.95 ± 0.04	1300 ± 0.00	0.006 ± 0.0015
3	15.0	7.64 ± 0.005	8.72 ± 0.22	1.81 ± 0.09	1375 ± 25	0.006 ± 0.001

Cont /...

Table 6.1 (Continued)

	NOMINAL CONCENTRATIONS OF AMMONIA*1			MEASURABLE CONCENTRATIONS OF AMMONIA*2		
				Diluted Water Samples		Undiluted Water Samples*3
	NH_4Cl mgl^{-1}	Total Ammonia-N mgl^{-1}	NH_3-N mgl^{-1}	Total Ammonia-N mgl^{-1} Mean $\pm$ SE	NH_3-N mgl^{-1}	Total Ammonia-N
Control	0.00	0.00	0.00	0.673 $\pm$ 0.004	0.008	0.00 (-ve ABS)
1	88.551	23.19	0.261	2.843 $\pm$ 0.572	0.032	0.00 (-ve ABS)
2	124.122	32.50	0.382	4.438 $\pm$ 0.69	0.052	0.00 (-ve ABS)
3	159.420	41.74	0.492	3.143 $\pm$ 1.09	0.037	0.00 (-ve ABS)

*1 Nominal values of total and unionized ammonia were calculated based on the mgl^{-1} NH_4Cl added to the test solutions

*2 Measurable concentrations of ammonia were erratic in presence of tris buffer, when diluted water samples were used

*3 Undiluted water samples resulted in negative absorbances (-ve ABS), due to the lack of colour development

Table 6.2 Concentrations of ammonium chloride and ammonia used in the test solutions and other measured physico chemical characteristics (trial 2 trout experiment) mean ± SE, n = 2

	Temperature °C	pH Mean ± SE	Dissolved Oxygen mgl-1 Mean ± SE	Total Alkalinity meq l^{-1} Mean ± SE	Electrolyte Conductivity nS cm^{-1} Mean ± SE	Total Hardness mgl^{-1} Mean ± SE
Control	15.0	7.87 ± 0.03	9.08 ± 0.075	1.34 ± 0.49	1690.0 ± 0.00	243.25 ± 2.25
1	15.0	7.84 ± 0.005	8.85 ± 0.15	1.66 ± 0.20	1787.5 ± 12.5	249.5 ± 3.50
2	15.0	7.84 ± 0.025	9.175 ± 0.075	0.86 ± 0.013	1835.0 ± 25.0	246.75 ± 0.75

Cont /...

Table 6.2 (Continued)

	NOMINAL CONCENTRATIONS OF AMMONIA*1			MEASURABLE CONCENTRATIONS OF AMMONIA*2		
				Diluted Water Samples		Undiluted Water Samples
	NH_4Cl mgl^{-1}	Total Ammonia-N	NH_3-N mgl^{-1}	Total Ammonia-N mgl^{-1} Mean ± SE	NH_3-N mgl^{-1}	Total*3 Ammonia-N
Control	0.00	0.00	0.00	1.946 ± 1.06	0.039	0.00 (-ve ABS)
1	56.23	14.72	0.292	6.90 ± 1.11	0.128	0.00 (-ve ABS)
2	78.73	20.61	0.40	6.85 ± 0.27	0.127	0.00 (-ve ABS)

*1 Nominal values of total and unionized ammonia were calculated based on the mgl^{-1} NH_4Cl added to the test solutions

*2 Measurable concentrations of ammonia were erratic in presence of tris-buffer when diluted water samples were used

*3 Undiluted water samples resulted in negative absorbances (-ve ABS) due to the lack of colour development

Plate 39.

A SEM of control trout gills after 7 days experimentation in low hardness tris-buffered experiments showing normal appearance of the filaments and lamellae, 260X.

Plate 40.

a. A SEM of trout gill exposed to 0.48 mgl^{-1} UIA after 3 days exposure showing chloride cell proliferation onto the lamellae from the afferent side of the filament, while the efferent side of the filament appears smooth and devoid of chloride cells, 400X.

b. The efferent side of the gill lamellae showing no chloride cells on the lamellae 1300X.

c. The efferent side of the filament and lamellae showing chloride cell proliferation taking place selectively, 1100X.

a

b

c

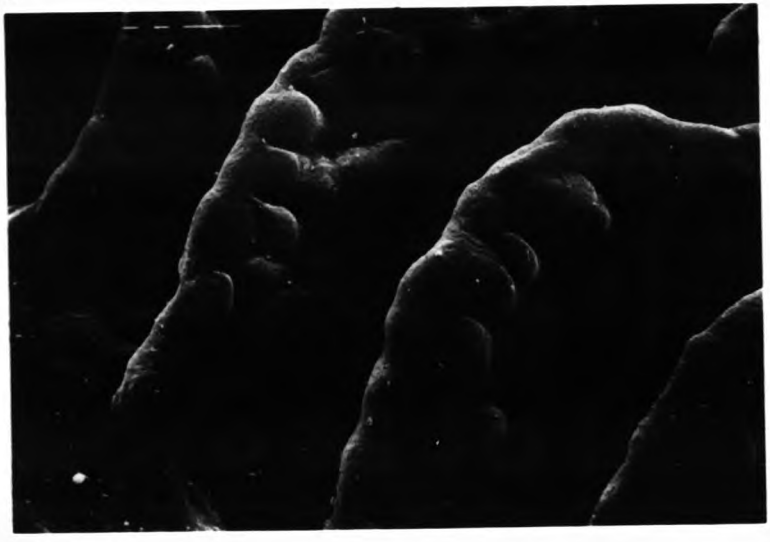


Plate 41.

SEM of trout gill filament tip from fish exposed to 0.48 mgl^{-1} UIA for seven days, exhibiting severe exhaustion of the mucous cells and hypertrophy, 800X.

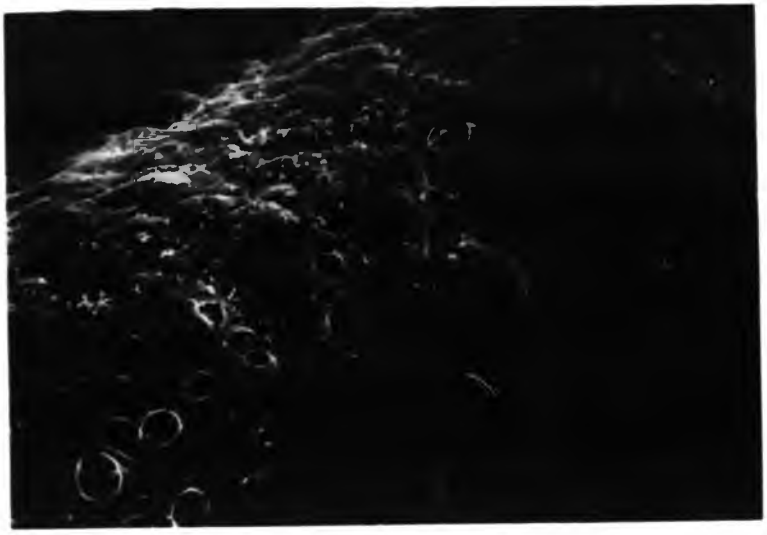


Plate 42.

Photomicrographs of the mucous cell changes noted at different ammonia treatments in low hardness tris-buffered experiments.

a. Control trout gill arch epithelium showing normal mucous cells in multiple layers. (PAS/AB at pH 2.5), 375X.

b. Exhaustion and vacuolation of mucous cells of the trout gill arch epithelium at 0.38 mgl^{-1} UIA after 7 days. Note the flocculated contents of the mucous cells (arrowed) (PAS/AB at pH 2.5) 600X.

c. Severe vacuolations and exhaustion of the mucous cells on the gill arch epithelium after seven days exposure to 0.49 mgl^{-1} UIA. PAS/AB at pH 2.5, 600X.

a

b

c

6.3.3.2.HISTOPATHOLOGY(TRIAL- 2, HIGH HARDNESS EXPERIMENT)

The histopathology in the gills of fish subjected to ammonia in high hardness tris-buffered dilution water appeared more severe than the changes noted in the experimental trial 1, in which low hardness tris-buffered dilution water was used.

Although the control fish remained normal, an occasional increase in chloride cell numbers on the gill filament and lamellae was clearly seen (Plate 43).

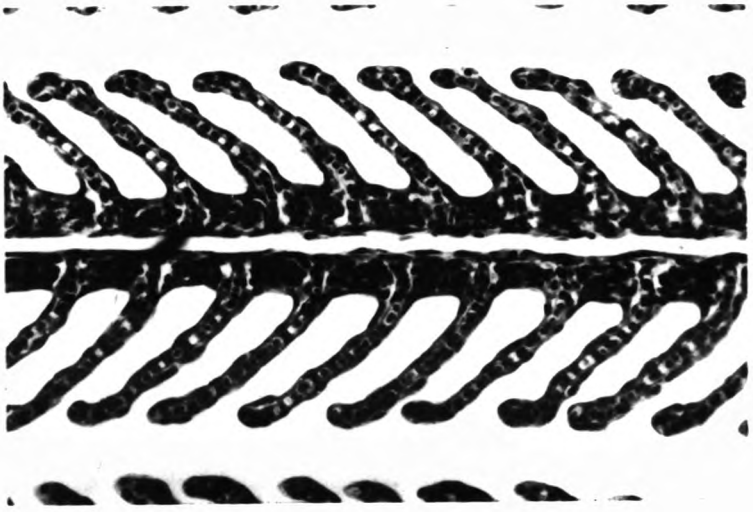
The basic lesion types noted in the ammonia exposed gills were the same as those already described. These lesions which included increased chloride cell proliferation, mucous cell necrosis and cellular oedema of the gill epithelia, remained moderately high in fish exposed to 0.29 mgl^{-1} UIA (Plate 44).In fish exposed to 0.40 mgl^{-1} UIA, the chloride cell hyperplasia and hypertrophy was severe and extensive but the most severe changes seen were the cellular vacuolations (Plate 45). These cellular vacuolations became bigger and extended on to the lamellae giving a foamy appearance to lamellar epithelia (Plate 46a). In addition to these changes, lifting of the lamellar epithelium was also noted occasionally (Plate 46b). However this particular reaction (*i.e.* lifting of the lamellar epithelium) was seen only in a few fish.The necrotic changes in the gill arch and filament epithelia were the most conspicuous (Plates 47 and 48).

Plate 43.

Photomicrographs of control trout gills from high hardness tris buffered experiments.

a) Normal filament and lamellae with very mild swelling of the lamellar epithelium after 3 days of experimentation. H&E, 375X

b) Control trout gill lamellae after 7 days of experimentation.
Note the appearance of chloride cells on the lamellae and moderate swelling (arrowed). H&E, 600X



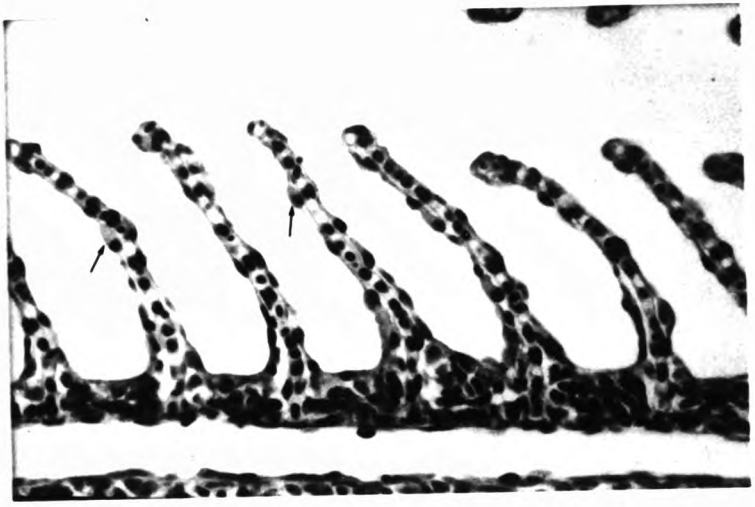


Plate 44.

Photomicrographs of the gills from trout exposed to 0.38 mgl^{-1} UIA for 7 days.

Note: severe chloride cell proliferation and necrotic changes.

H&E, 375X.

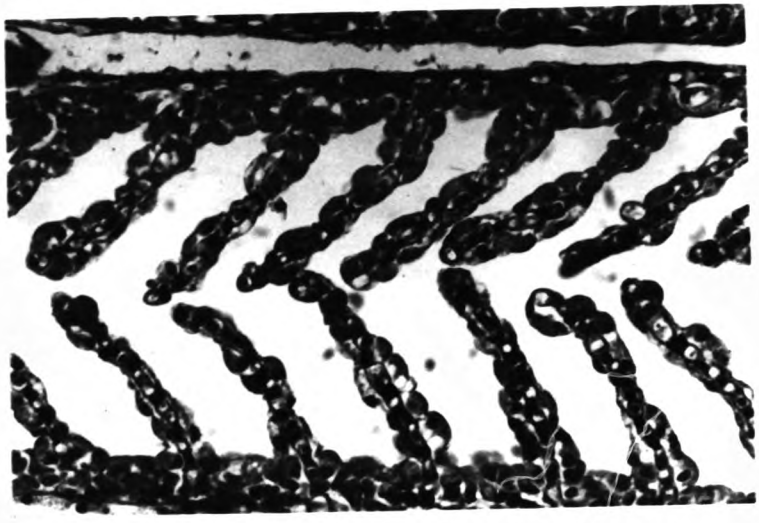


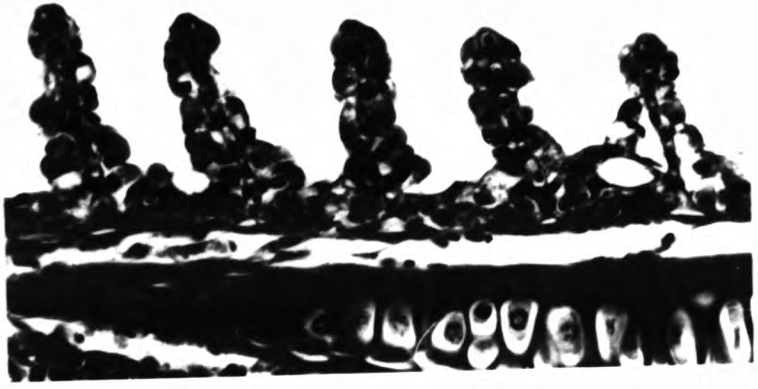
Plate 45.

A photomicrograph of trout gills from fish exposed to 0.49 mgl^{-1} UIA for 7 days.

a) Severe chloride cell proliferation and vacuolations. H&E 600X

b)Photomicrograph showing increased vacuolations and intra cellular oedema.

H&E 1500X



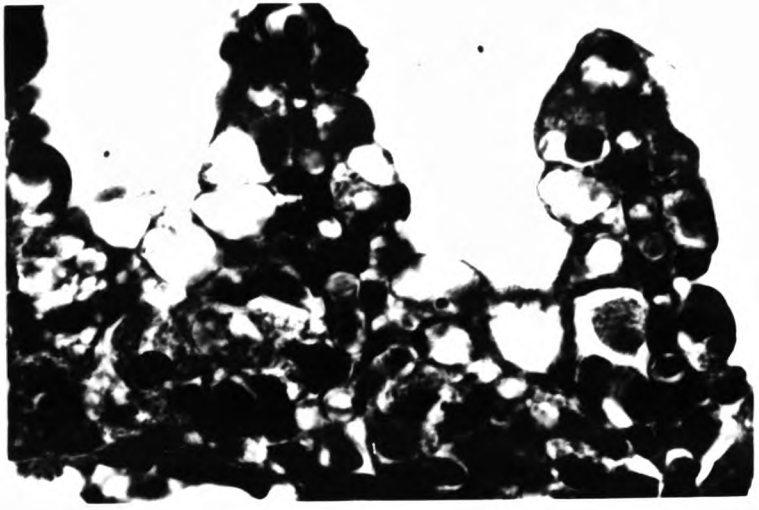
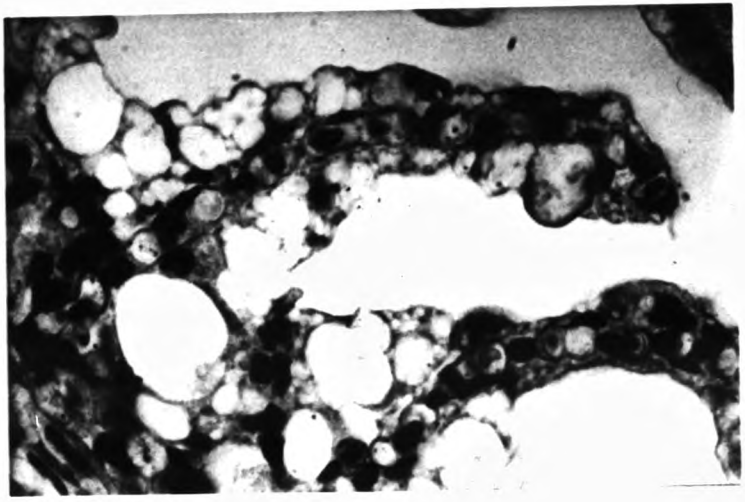


Plate 46.

a) A photomicrograph of trout gills exposed to 0.49 mgl^{-1} UIA for 7 days, showing extensive vacuolations and cellular oedema. 1500X

b) Separation of lamellar epithelium (epitheliocapillary separation) at 0.49 mgl^{-1} UIA from trout after 7 days exposure. 600X



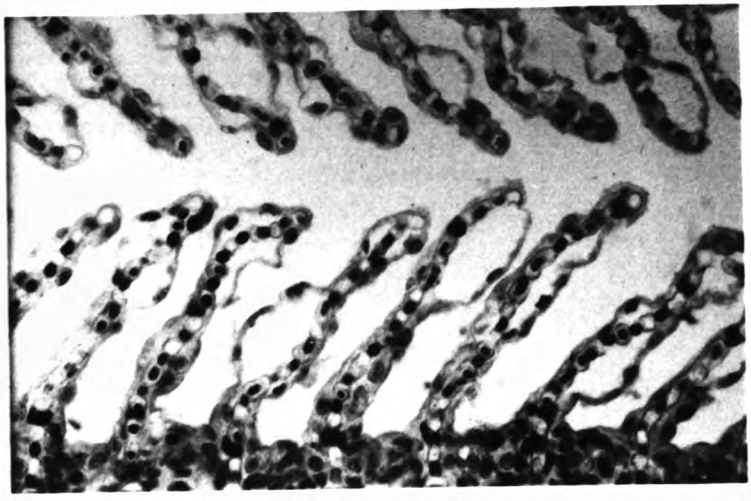
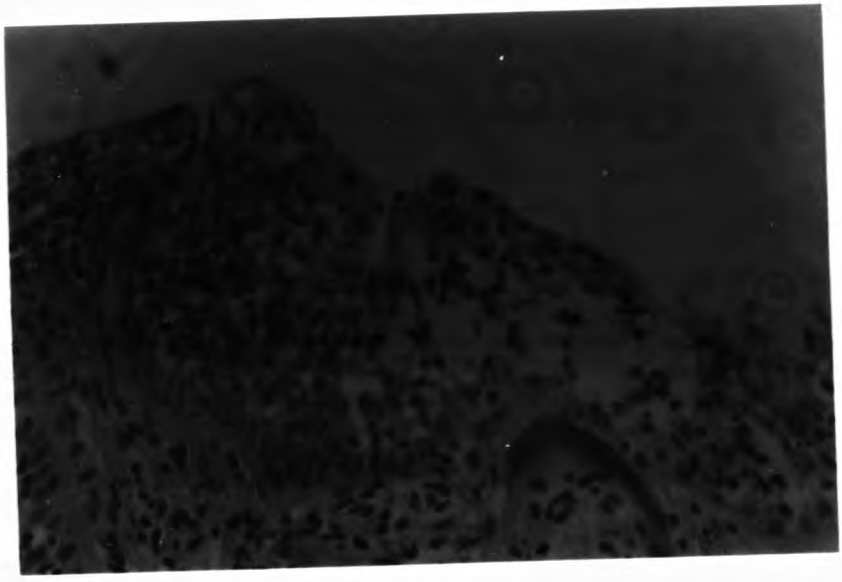


Plate 47.

a) Gill arch from trout exposed to 0.49 mgl^{-1} UIA for seven days, showing extensive vacuolations and cellular necrosis. H&E, 600X

b) A high magnification photomicrograph of the gill arch epithelium (same as above) showing pyknotic (small arrows) and karyorrhexis
(big arrows) of the nuclei with severe oedema. H&E, 1500X



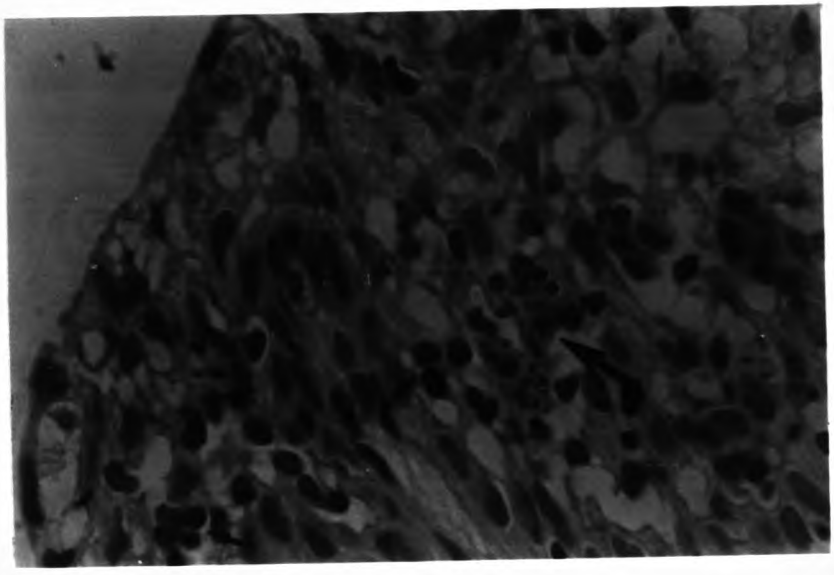


Plate 48.

Gill filament epithelium in the inter lamellar region of trout gills exposed to 0.49 mgl^{-1} UIA for seven days.

Note similar necrotic changes as seen in the gill arch pithelium. H&E, 600X

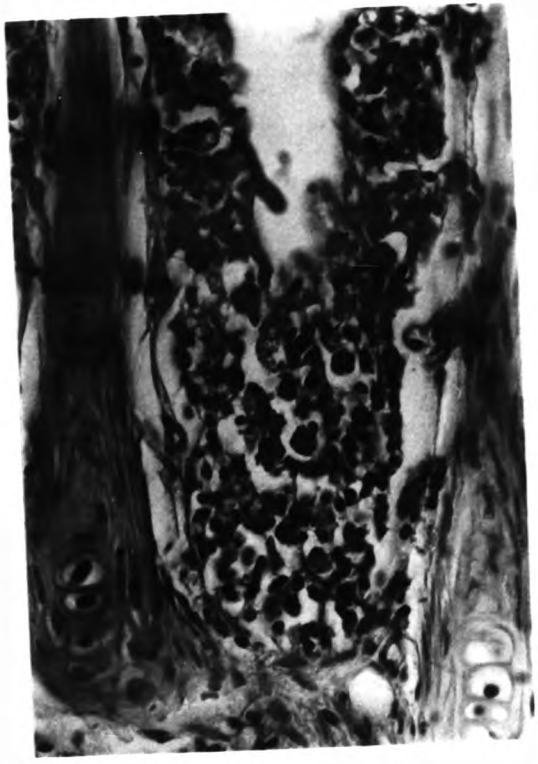
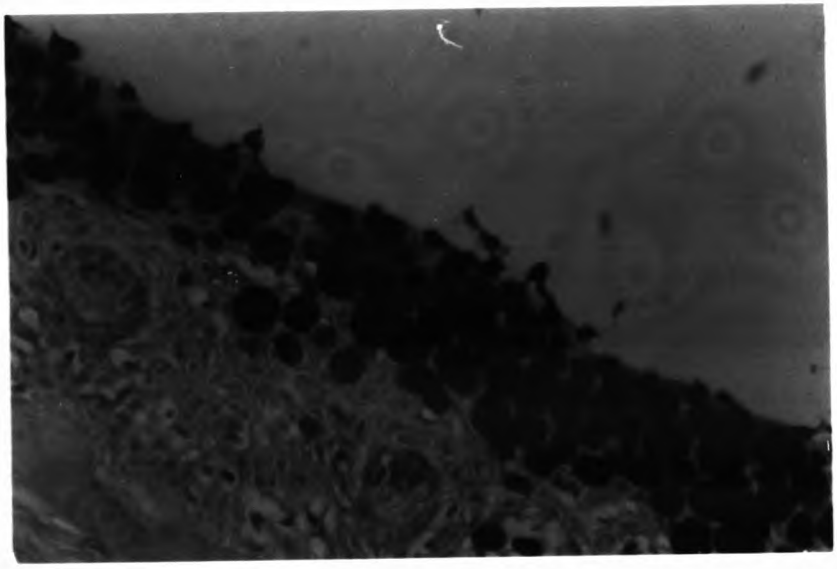


Plate 49.

Photo micrograph of the control trout gill arch epithelium showing normal mucous cells.

a) After 3 days experimental period, PAS, 600X

b) After 7 days experimental period, PAS, 600X



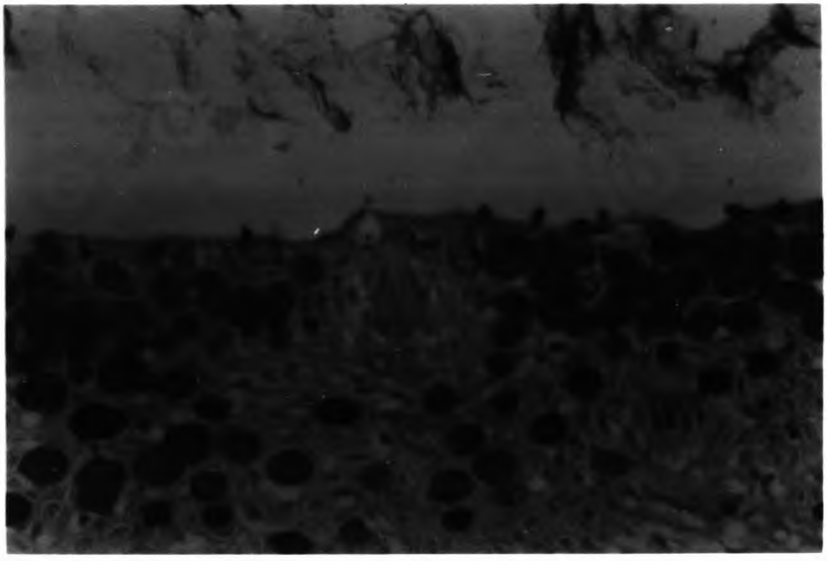
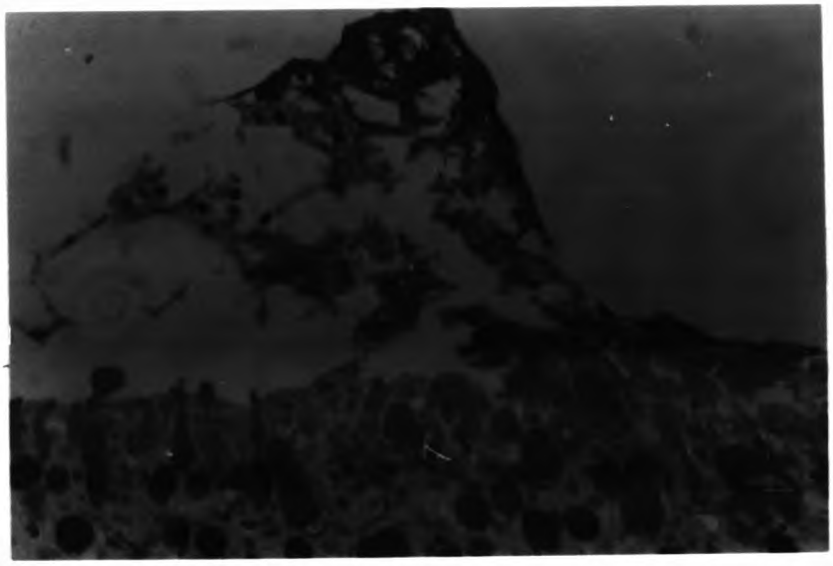


Plate 50.

a) Photomicrograph of the mucous cells from the gill arch epithelium of trout exposed to 0.29 mgl^{-1} UIA for 3 days showing increased mucus secretion and sloughing. PAS, 600X

b) After 7 days exposure. Note the increased mucous cell vacuolations in addition to oedema of the epithelium. PAS, 600X



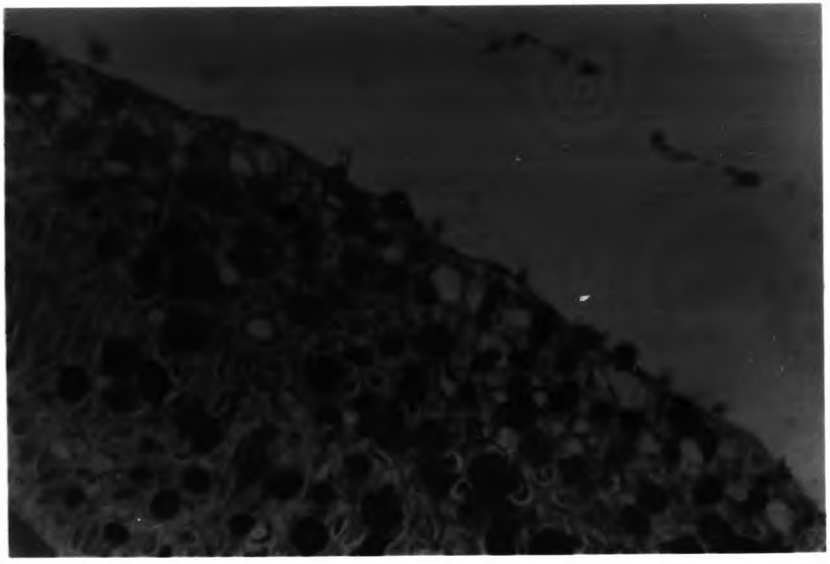
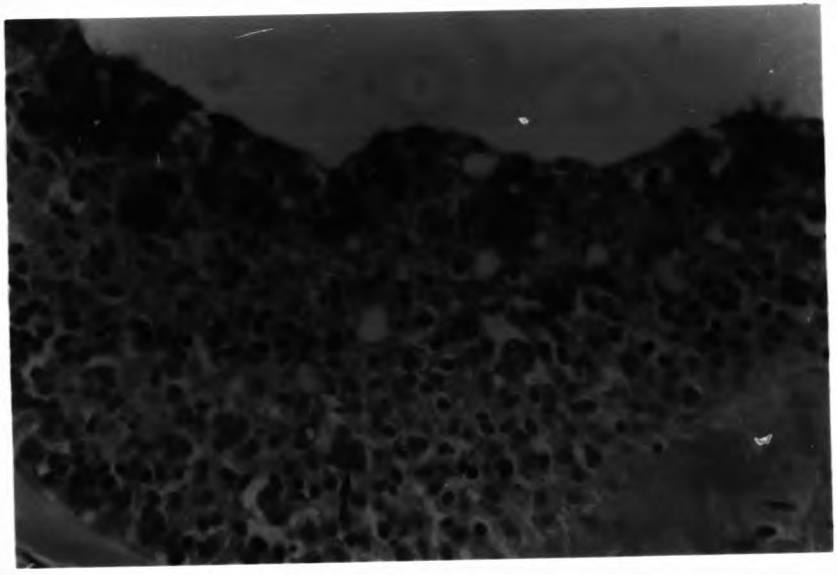


Plate 51.

a) A photomicrographs of trout gill arch epithelium at 0.49 mgl^{-1} UIA after three days exposure showing mucous cell necrosis and extensive nuclear pyknosis, and what appears to be the phagocytosis of such necrotic cells within the epithelium (arrowed). PAS, 600X

b) A photo micrograph of trout gill arch epithelium at 0.49 mgl^{-1} UIA showing extensive mucous cell vacuolations and necrosis of the epithelium after seven days exposure. PAS, 600X



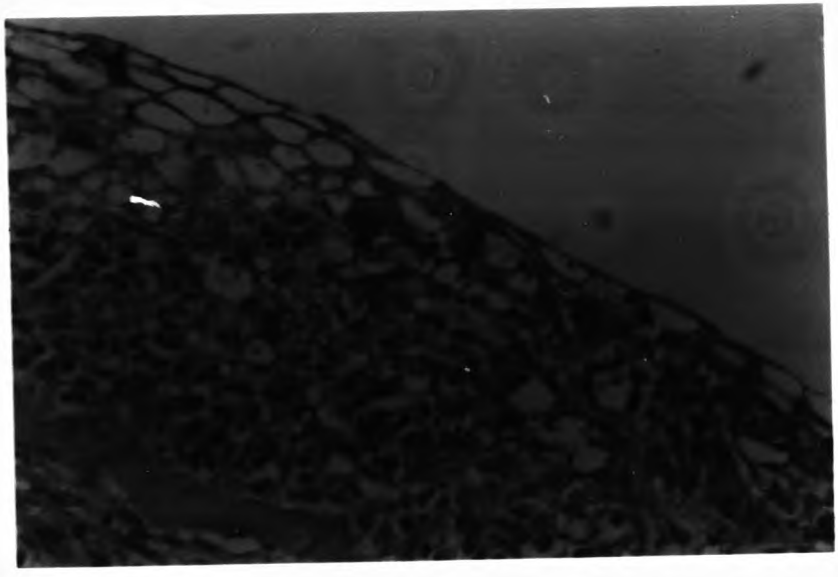


Plate 52.

Photographs of experimental trout exhibiting severe neurological distress after four days exposure.

a) At 0.42 mgl^{-1} UIA in high hardness tris-buffered dilution water.

b) At 0.49 mgl^{-1} UIA in low hardness tris-buffered dilution water.

Plate 53.

A photograph showing the colour development patterns in the tris-buffered and phosphate buffered water samples when analysed by the phenol hypochlorite method.

a) 0.5 mgl^{-1} UIA, in tris buffer.

b) 1.0 mgl^{-1} UIA in tris buffer.

c) 2.0 mgl^{-1} UIA in tris buffer.

d) 2.0 mgl^{-1} UIA in phosphate buffer.

e) 1.0 mgl^{-1} UIA in phosphate buffer.

f) 0.5 mgl^{-1} UIA in phosphate buffer.

These differences noted in the histology of the fish gills exposed to high ammonia in high hardness water from those changes noted in low hardness and high ammonia treatments appeared to be reflected in the fish mortalities in these two particular treatments. In the first trial where the concentration of ammonia was 0.481 mgl^{-1} UIA and the hardness of the water was low, no mortalities occurred as a result of continued exposure to ammonia (except for the initial two deaths due presumably to the sudden shock of exposure to high ammonia). Whereas in the case of trial 2 where the ammonia concentration was 0.40 mgl^{-1} as UIA (lower than the first trial) but the hardness of the water very high, the mortalities were not noticed until after 48 hours and more fish started to die after day 3, while at the same time the rest of the fish exhibited signs of ill-health. These observations, in combination with severe epithelial oedema and lifting, indicate the probable additional stress as a consequence of increased hardness in addition to high ammonia in trial 2.

DISCUSSION

TROUT EXPERIMENTS

The control trout gills once again did not show any recognizable pathological alterations in their structure after 7 days exposure to 0.01 M tris-buffered dilution water in both trials 1 and 2, although an increased numbers of chloride cells were noted in trial 2.

Similarly ammonia in tris-buffered experiments did not produce

any difference in histopathology, particularly in the low hardness experiments, from that already noted in phosphate buffered ammonia exposure experiments, indicating that the toxicity of ammonia was the same in both tris-buffered and phosphate buffered-water. The characteristic cellular changes of trout gill reaction to ammonia, such as the chloride cell proliferation and hypertrophy, mucous cell necrosis, inter and intra cellular oedema (cellular vacuolations), were the common changes noted in these experiments also.

However, the histopathological changes noted in the trout exposed to ammonia in high hardness water (trial 2) was much more severe. This increased damage also appeared to be reflected in the fish's survival as more fish continued to die, further indicating that the ammonia was more toxic in the case of high hardness water than in low hardness water.

The filamental and lamellar epithelial vacuolations increased greatly with the epithelium appearing increasingly swollen and foamy in appearance . Fewer stainable mucous cells were seen amongst severely vacuolated (exhausted) mucous cell populations and such mucous cell degeneration increased from low to high ammonia concentrations (Plates 50 and 51).

Since the difference between the experimental procedures from trial 1 to trial 2 was only an eight fold increase in hardness, this appears to have exacerbated the effect on fish particularly at high ammonia concentrations, indicating a probable effect of an increased ionic load at the gill surface.

Although variation in hardness has not been known to have a

significant effect on the LC 50 values for fish in terms of unionized ammonia (Emerson *et al.* 1975; Alabaster & Lloyd 1980), nevertheless Tomasso *et al.* (1980) have suggested that the differences in the environmental hardness and pH of the experimental media were the primary causes of a wide range in reported ammonia LC 50 values to fish.

Variations in the toxicity of various pesticides to different fish species as a result of an increase or decrease in hardness have also been reported. Murthy (1986 b), summarising the effects of hardness on the toxicity of pesticides from the reported literature, noted that a majority of the studies found no influence of hardness on the toxicity, while a considerable numbers of other reports indicated an increase in the toxicity at high hardness (references cited by Murthy 1986 b). Some of these studies have also indicated variations in both pH and hardness as responsible factors for the increased toxicity. Therefore, Murthy (1987b) has suggested that perhaps it would be worthwhile to have a fresh look at this problem by testing the influence of pH and hardness separately and together, in the case of all those pesticides supposed to have been influenced by hardness.

In the present experimental trials on trout the pH was adjusted by tris-buffer but the final mean pH values of the experiment were slightly higher in the case of the high hardness trial than in the case of the low hardness trial (Tables 6.1 and 6.2). Hence it is possible that both increased hardness and increased pH might have been responsible for the increased toxicity.

Although the exact mechanism of such increased toxicity of ammonia at high hardness remains less clear, it is possible that in the presence of an already existing toxic ammonia load, an eight fold increase in the external ionic load might have resulted in the disturbance of branchial salt fluxes and the possible upset of the cellular ionic balance and hence the normal cellular metabolism. The increased pyknosis of the cells in the epithelium in general and increased vacuolar swellings of cells (Plates 47 and 48) of the lamellar and filamental epithelium observed may reflect such damage.

Since hardness is, practically, a measure of calcium and magnesium ions, an increase in hardness would obviously have resulted in a proportionate increase in the concentration of calcium and magnesium. From the available literature based on recent studies it has also become apparent that branchial permeability to Na^+ and Cl^- is affected directly by the external calcium concentration (a depletion of calcium by transfer of fish to distilled or deionized water resulting in the loss of body Na^+ and an enrichment of external calcium resulting in reduced permeability of the gill membrane to Na^+ and to a lesser extent to chloride (Cuthbert & Maetz 1972; Randall., Perry and Hemming, 1982; MacDonald, 1983). Thus it is possible that elevated calcium levels in high hardness water might have impeded the essential exchange processes such as Na^+/NH_4^+ (H^+) and Cl^-/HCO_3^- thus increasing the cellular toxicity of ammonia. Since magnesium is also known to behave similarly to calcium (Rankin and Davenport 1981) it is possible that cellular permeability might have been greatly affected.

However, the possibility of tris-buffer interacting or

competing with elevated levels of other ions present in high hardness water and with that of ammonia itself needs further investigation.

In the tris-buffered experiments the fish were neurologically more affected than in the case of phosphate buffered experiments at similar levels of ammonia. This in other words suggests a possibility of alternative ways by which tris-buffer may be interacting in the presence of ammonium ions with the physiological activities of the fish. Also it is not clear as to the exact mechanism of interference of tris with ammonia measurement. Whether the interaction is with the ammonia present with in the water or with those of the chemical used in the measurement of ammonia is not known. Since such interference was still present when the tris-buffered water containing ammonia was diluted a 100 fold in distilled water, it appears that tris- buffer may intact be interacting with ammonia directly.

Rice & Stokes (1975), who used tris-buffer at 0.05 M concentration, which was five times higher than the concentration used in the present trials, and measured ammonia by nesslerization method recorded the lowest LC 50 values for adult rainbow trout so far known in the literature. Their LC 50 value (recorded as 24-h, TLM Value) for adult trout at a temperature value of 10°C and pH 8.3 was only 0.097 mgl^{-1} as UIA. This value is at least 5 to 6 times lower than the recorded LC 50 values for trout by various other authors range between 0.47 - 0.70 mgl^{-1} as UIA (Alabaster & Lloyd 1980). This difference definitively seems to be due to the misleading interference of tris-buffer in the ammonia measurements.

However, Alabaster & Lloyd (1980), referring to the TLM values of Rice and Stokes (1975), have commented as follows, "the pH value of the water in these experiments was controlled by the addition of tris-buffer, and this procedure may have affected the toxicity of ammonia", the exact mechanism involved remains unknown needing further investigations.

In conclusion the tris-buffered ammonia toxicity experiments on trout can be summarized as follows.

1. Tris-buffer as such did not bring about any histological changes in the trout gill structure, which is in agreement with the observations noted in phosphate buffered control treatments.

2. The histopathology due to ammonia in tris-buffered experiments did not differ from that of the phosphate buffered experiments under normal conditions of water hardness.

3. But elevation in the hardness of tris-buffered water, appears to increase the toxicity of ammonia along with the associated tissue damage.

4. In general the rainbow trout appear to be neurologically more severely affected in tris-buffered high ammonia treatments than their counterparts in phosphate buffered dilution water.

EXPERIMENTS WITH CARP

6.5.MATERIALS AND METHODS

Two experimental trials were carried out on carp in tris-buffered dilution water at different levels of unionized ammonia. The experimental method and procedures followed were identical to those used in Chapter 4 except for the use of tris-buffer at 0.01 M concentration to maintain the pH. Two groups of fish were used. In the first trial the fish used were smaller and weighed 4.42 $\pm$ 1.99 grams(± SD) and measured 6.96 $\pm$ 1.80 cms,(± SD) while those used in the second trial weighed 52.72 $\pm$ 11.46 grams(±SD) and measured 13.53 $\pm$ 1.67 cms(±SD).

6.6 RESULTS

6.6.1 WATER QUALITY

Mean values of pH and temperature along with the various unionized ammonia levels used in trial 1 and trial 2 are given in Table 6.3 and 6.4, respectively. Because of the interference of tris-buffer with the measurement of ammonia the measurable values were erroneous and very variable. Although attempts were made to adopt an alternative method (direct Nesslerization method; Stirling, 1985) wide variations were encountered. Hence total ammonia levels were calculated from the exact amounts of ammonium chloride added to the water and the same values were used in calculating the unionized ammonia levels based on the measured mean values of pH and

temperature during the experimental period. Since all the fish used in these experiments were starved for at least one week before experimentation it was expected that the increases in the ammonia levels as a result of the fish's own metabolic activity (especially in the controls) were negligible as already noted in Chapters 3 and 4 for carp and trout respectively.

6.6.2.MORTALITIES

Thirty percent of the carp died in ammonia concentrations of 1.72 mgl^{-1} in the second trial. Except for this no mortalities were noted in other experimental treatments.

Table 6.3 Concentrations of ammonium chloride and ammonia used in the test solutions and other measured physico-chemical characteristics (trial 1, carp experiment)*1

	NH_4Cl mgl^{-1}	Total Ammonia-N*2 mgl^{-1}	NH_3-N*2 mgl^{-1}	Temperature ^oC Mean ± SE	pH Mean ± SE	Dissolved oxygen mgl^{-1} Mean ± SE	Total Alkalinity $meql^{-1}$ Mean ± SE
Control	0.00	0.00	0.00	28.20 ± 0.22	7.56 ± 0.042	7.05 ± 0.15	2.94 ± 0.68
1	38.22	10.007	0.23	28.05 ± 0.36	7.52 ± 0.025	8.1 ± 0.9	2.04 ± 0.4
2	76.43	20.014	0.42	27.58 ± 0.46	7.50 ± 0.051	7.7 ± 0.8	2.09 ± 0.71

*1 The mean hardness of the dilution water used in this trial was 66.33 (SD, 1.24, n=6) mgl^{-1} as $CaCO_3$

*2 Total and unionized ammonia concentrations were calculated from the mgl^{-1} NH_4Cl added to the test solution

Table 6.4 Concentrations of ammonium chloride and ammonia used in the test solutions and other measured physico-chemical characteristics (trial 2, carp experiment)*1 Mean ± SE, n = 2

	NH_4Cl mgl^{-1}	Total Ammonia-N*2 mgl^{-1}	NH_3-N*2 mgl^{-1}	Temperature ^oC Mean ± SE	pH Mean ± SE
Control	0.00	0.00	0.00	26.47 ± 0.37	7.74 ± 0.08
1	64.50	16.886	0.607	27.23 ± 0.57	7.75 ± 0.05
2	193.29	50.603	1.724	26.42 ± 0.61	7.75 ± 0.05

*1 The mean hardness of the dilution water used in this trial was 52.6 (SD, 1.62, n=6) mgl^{-1} as $CaCO_3$

*2 Total and unionized ammonia concentrations were calculated from the mgl^{-1} NH_4Cl added to the test solutions

Table 6.5 A list of the diverse buffer chemicals and concentrations that have been adopted by various workers in ammonia toxicity studies

Author	Fish Species	Nature of Study	Buffer Compostion and concentration	Comments
1. Downing and Merkens, 1955	Rainbow Trout *Salmo gairdneri*	Effects of dissolved oxygen on ammonia	A mixture of NaOH and NH_4Cl Concentration unknown	No detrimental effects reported
2. Sousa, *et al.*, 1974	Chinook Salmon *Onchorhynchus tshawtyscha*	Ammonia toxicity	Monosodium phosphate and sodium bicarbonate Concentration unknown	No detrimental effects reported.
3. Smart, 1975	Rainbow trout *Salmo gairdneri*	Ammonia toxicity	1.0N NaoH or 1.0N HCl at appropriate concentrations	No detrimental effects reported.
4. Rice and Stokes, 1975	Rainbow trout *Salmo gairdneri*	Acute ammonia toxicity	Tris - buffer 0.05M concentration	No apparent detrimental effects identified but recorded the LC50 values lowest ever reported.
5. Robinette, 1976	Channel Catfish *Ictalurus punctatus*	Sublethal effect of ammonia on growth	A combination of sodium bicarbonate and ammonium chloride	No detrimental effects reported

Cont /...

Table 6.5 (Continued)

Author	Fish Species	Nature of Study	Buffer Compostion and concentration	Comments
6. Rubin and Elmaraghy, 1977	Guppy *Poecilia reticulata*	Ammonia toxicity study	A combination of 0.5M potassium phosphate and 0.2M potassium nitrate at 0.001M as phosphate	No detrimental effects reported.
7. Buckley, 1978	Coho salmon *Onchorhynchus kisutch*	Acute toxicity of ammonia	Boric acid buffer 0.004M	No detrimental effects reported
8. Alderson, 1979	Dover Sole, *Solea solea (L)* and Turbot, *Scopthalmus maximus*	Effects of ammonia on growth	Sodium hydroxide (NaOH) or carbon dioxide. Concentration unknown.	No detrimental effects reported
9. Brownell, 1989	Marine fish larvae	Ammonia tolerance limits	Sodium hydroxide and sodium bicarbonate ($NaHCO_3$). Concentration unknown.	No detrimental effects reported
10. Tomasso, *et al.*, 1980	Channel Catfish	Effects of pH and calcium on ammonia toxicity	Potassium phosphate as KH_2PO_4 and K_2HPO_4 at 0.01M concentration.	No detrimental effects.

Cont /...

Table 6.5 (Continued)

Author	Fish Species	Nature of Study	Buffer Compostion and concentration	Comments
11. Harder and Allen, 1983	Chinook Salmon	Ammonia toxicity	Sodium bicarbonate concentration unknown.	No detrimental effects reported.
12. Mitchell and Cech, 1983	Channel Catfish	Effects of ammonia on gills	A 10M sodium hydroxide was used. Final concentration unknown.	No detrimental effects reported.
13. Sheehan and Lewis, 1986	Channel Catfish	Influence of pH and ammonium salts on the toxicity of ammonia	Mixtures of, mono-, di-, and tri basic phosphate buffers.	No detrimental effects reported.
14. Hasan and Macintosh, 1986	Common Carp Cyprinus carpio	Ammonia tolerance limits.	A combination of sodium dihydrogen phosphate (NaH_2PO_4) and di-sodium hydrogen phosphate (Na_2HPO_4) at 0.01M concentration.	No detrimental effects reported.

6.6.3.HISTOPATHOLOGY

CONTROL FISH (TRIAL 1 AND 2)

Typical lesions of phosphate buffered experimental control carp gills (Chapter 4) were not seen in carp subjected to tris-buffered dilution water. There were no fibroblast like elongated cells seen in the lamellar and inter lamellar regions. In otherwords the control gills remained normal (Plate 54).The EGCs were moderately present throughout the gill tissues. Rodlet cells were also present especially in the connective tissues of gill septum and arch.

FISH EXPOSED TO LOW AMMONIA

Fish subjected to the lowest ammonia concentration of only 0.23 mgl^{-1} UIA did not show any changes but for the presence of some of the lamellar alterations seen in the control fish. But fish subjected to the other two low levels of ammonia (ie 0.42 and 0.61 mgl^{-1} UIA) showed mild but significantly recognisable swellings of the kind noted in the phosphate buffered ammonia exposed carp (Plate 55).

In these two low level ammonia concentrations the frequency of these lesions that were typically nodular swellings, were smaller in size (Plate 55). The chloride cells on the inter lamellar epithelium were and conspicuously swollen, relatively fewer chloride cells were noted on the lamellae. There was an increase in the density of EGCs when compared with the controls in the epithelia as well as the connective tissues.

AT HIGH AMMONIA

At a high ammonia concentration of 1.72 mgl^{-1} UIA (trial 2), the lesions were typically identical to those already seen in high ammonia exposed carp in phosphate buffered water. The nodular swelling were extensive on the filament and arch epithelium (Plate 56,58a). Large intact sloughed nodules were seen in the interfilamental areas. Aggregations of EGCs enclosed within syncytia like structures along with other necrotic cells were extensive seen (Plate 56). In these bigger fish, the chloride cells were severely hypertrophied on the lamellae and appeared in small aggregations (Plate 67). Condensation of the nuclear material (pyknosis) and cellular necrosis was also noted in such cells. Necrosis of the epithelia in general was characteristic. Severe exhaustion of the mucous cells was also once again seen , while SEM also revealed the thickening of the mucus in the mucous cell openings in some areas of the gill epithelium.

The changes that were noted in EGCs and rodlet cells were similar to those in the phosphate buffered experiments.An increase in the rodlet cell infiltration was evident at low ammonia concentrations (Plate 59). The mucous cells on the filaments in the case of ammonia exposed carp were more PAS positive in combined AB and PAS staining at pH 1.0, indicating the predominance of carboxylated and neutral mucopolysaccharides.

Plate 54.

SEM of control carp gills, after seven days experimentation in tris-buffered dilution water, showing normal gill structure. 90X.

Plate 55.

a. SEM of carp gills exposed to 0.61 mgl^{-1} UIA for 7 days in tris buffered dilution water showing the appearance of nodular swellings on the filament epithelium (arrowed) 120X.

b. A higher magnification micrograph showing swelling of the epithelium with an increased mucous cell openings. 332X.

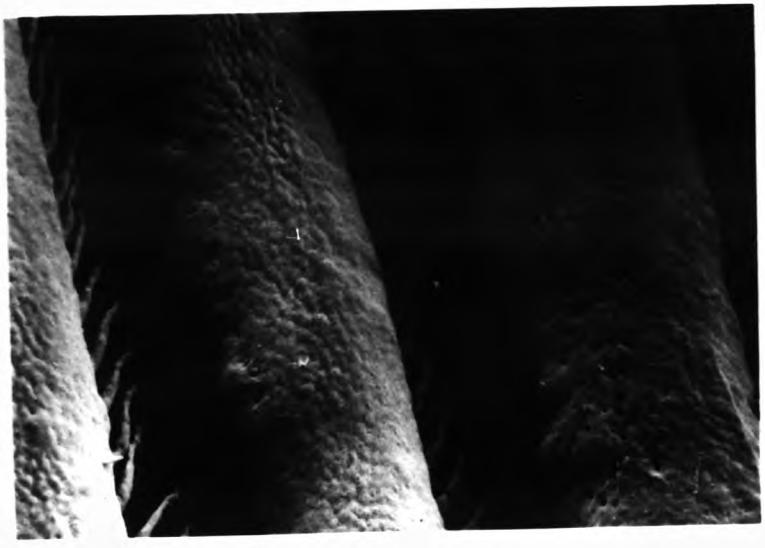
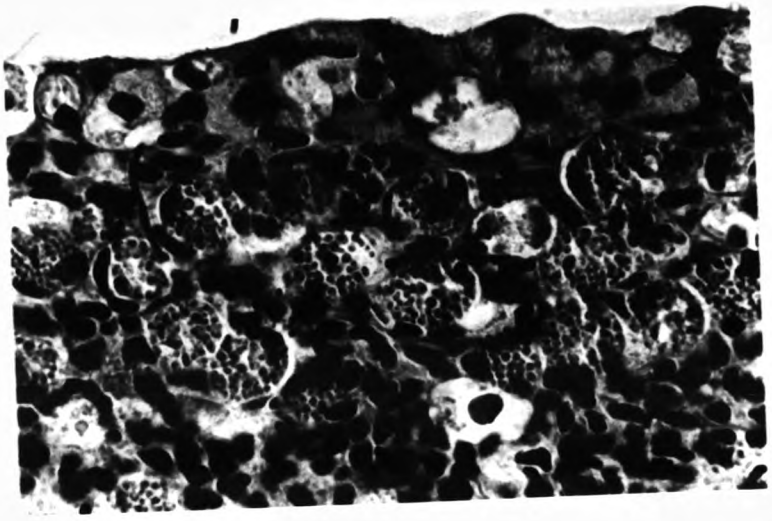


Plate 56.

Photomicrographs of carp gill epithelium from fish exposed to 1.72 mgl^{-1} UIA for 3 days.

(a) intensive infiltration of EGCs into the filament epithelium. H&E 1500X

(b) Developing nodular swellings of the epithelium. H&E, 1500X.



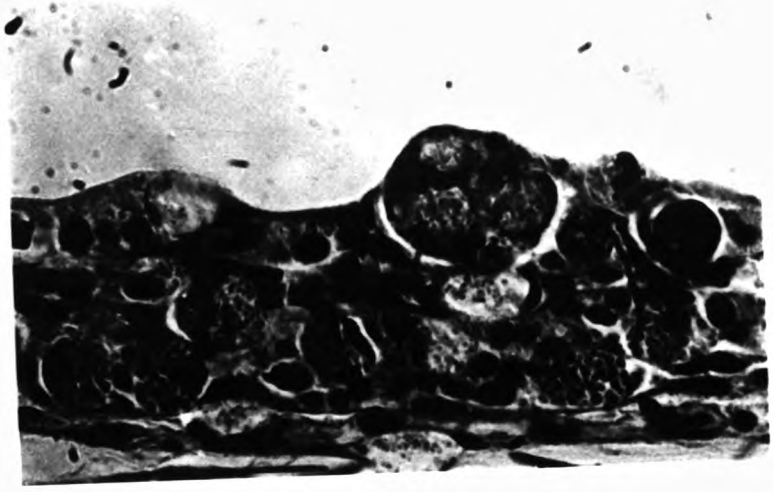


Plate 57.

Photomicrograph showing extensive chloride cell proliferation on the lamellae of carp

a. After three days exposure to 1.72 mgl^{-1} UIA.

b. After 7 days exposure to 1.72 mgl^{-1} UIA. H&E 600X.

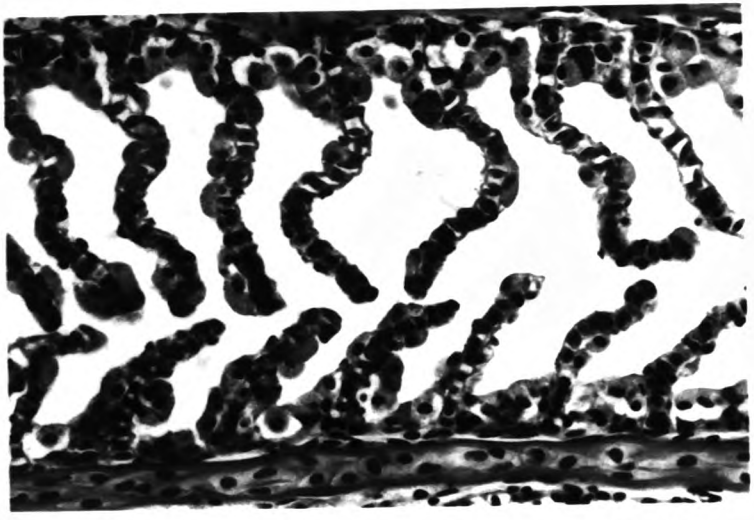


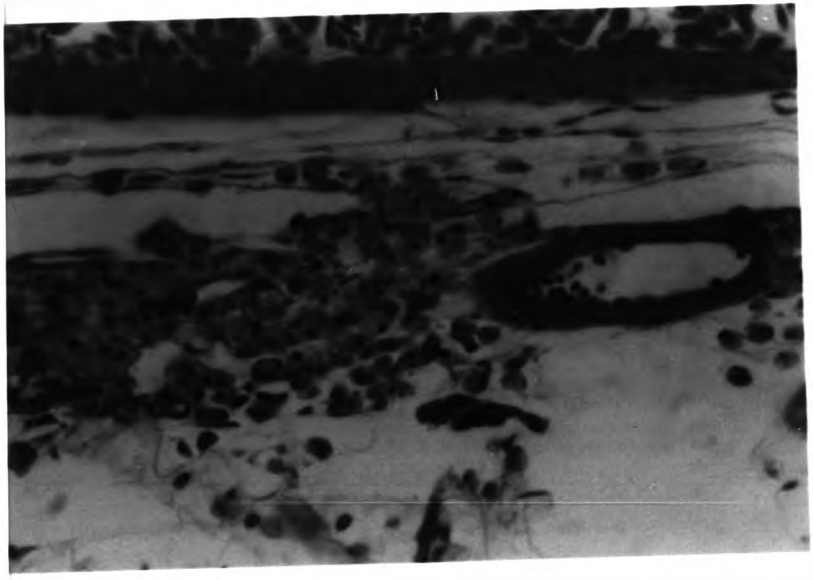
Plate 58.

a. Carp exposed to 1.72 mgl^{-1} UIA showing nodular swellings on the arch epithelium after three days exposure. PAS 600X.

b. SEM of the filament epithelium from carp exposed to 1.72 mgl^{-1} UIA for seven days showing the erosion of microridges on the filament epithelial cells and the thickened mucus on the mucus cell openings 2700X.

Plate 59.

A photomicrograph of the carp gill arch connective tissue showing an increased number of rodlet cells at 0.61 mgl^{-1} UIA after seven days exposure. Methylene blue/Basic fuchsin 600X.



DISCUSSION

CARP EXPERIMENTS

The current experimental finding that the control carp did not show any adverse histological alterations except for few minor changes, is intact in contrast to the observations made in the phosphate buffered experiments and it clearly demonstrated that tris-buffer was not as detrimental as phosphate buffer. However, its interference in the measurement of ammonia was once again its main disadvantage.

The minor changes that were noted in the control carp indicate that although tris-buffer is not so detrimental as phosphate buffer, nevertheless these appears to be some mild irritation being exerted on the gill epithelium.

In the case of fish exposed to 0.23 mgl^{-1} UIA, although there were no apparent lesions, at 0.42 mgl^{-1} UIA small lesions were clearly evident and such lesions moderately increased at 0.61 mgl^{-1} UIA but became more extensive and severe at 1.72 mgl^{-1} UIA. Thus these results showed a sharp difference in the concentration of ammonia at which the lesions were initiated compared with those of the phosphate buffered experimental results. In the previous carp experiments, no tissue lesions or alterations could be seen at ammonia concentrations as high as 0.50 mgl^{-1} UIA while 1.0 mgl^{-1} UIA showed only mild to moderate lesions, but here in the current experiments the lesions were present at ammonia concentrations of 0.42 and 0.61 mgl^{-1} UIA.

Hence these results indicate that the ammonia was more toxic in tris-buffered water than there of phosphate buffered waters. Since buffering of water with sodium phosphate buffer usually results in elevated levels of Na^+ ions, this might have been some how alleviated the toxic effect of ammonia (Redner *et al.* 1980) on the gill tissues at these low levels of ammonia. McFarland and Norris (1958) in their tris-buffered fish transportation experiments encountered variations in the measured ammonia concentrations, although these authors indicated that the method of ammonia estimation adopted here (Kjeldal method) is subjected to an error of at least 10 percent, nevertheless suggested that the $-NH_2$ group present in the buffer molecule might have dissociated during the process of estimation and resulted in the increases of ammonia concentration. If it is so the possibility of such dissociation occurring within tissue compartments or within the water may have to be considered.

Nevertheless the fact that the tissue lesions typical of ammonia exposure (as noted in the experiments detailed in Chapter 4.) increased with increasing concentrations of ammonia and that these lesions basically did not differ from the previous experiments has further demonstrated that the such cellular and tissue lesions as noted in these carp gills were a direct cause of ammonia toxicity.

Although extensive studies have been carried out in the past to understand the movement and fluxes of such ions as Na^+, NH_4^+. H^+ and Cl^-, what effect can these ions could have on the presence f or active fluxes of other ions such as HPO_4^{2-}, SO_4^{2-}

or various organic cations and anions (such as those of tris) have never been taken into account and this aspect certainly needs more attention (Heisler, 1984).

CHAPTER-7

EFFECT OF AMMONIA ON THE GILL STRUCTURE OF CARP AND TROUT IN UNBUFFERED DILUTION WATER.

7.1 INTRODUCTION

From the experimental results in chapter four and five, it was noted that the use of either phosphate or tris-buffer in itself did not result in any apparent histological changes in the case of rainbow trout (controls) and the pathology attributable to ammonia remained similar under both tris and phosphate buffered experimental conditions (except in the case of very high hardness: see chapter 6).

However, in the case of carp, although the tissue lesions due to high ammonia exposure remained the same in both phosphate and tris-buffered experiments, the phosphate buffer exerted severe detrimental effects which were unique to the control fish. Secondly, in the phosphate buffered experiments, the gill lesions were absent at 0.5 mgl^{-1} UIA, while in tris-buffered experiments mild lesions were evident both at 0.42 and 0.61 mgl^{-1} UIA. Thus these results indicated that some possible differences existed in the mode of action of ammonia in the presence of these two buffers, at least at the levels of low ammonia concentrations. These differences might have been due to the possible alleviation of ammonia toxicity at low levels of ammonia in phosphate buffered experiments or an exacerbation of the ammonia toxicity in tris-buffered experiments.

The fact that the rainbow trout exhibited more evere neurological signs in tris-buffered experiments than in the phosphate buffered experiments was also suggestive that ammonia was more toxic in tris-buffered experiments.

Hence, the possibility of tissue changes noted in carp and trout at high ammonia levels not having been influenced to certain extent by the buffers used could not be ascertained without the experimental knowledge of the effects of ammonia on the gills in the absence of these buffers. Therefore, a further set of experimental trials was carried out without the use of any buffers both on trout and carp to find out if ammonia in the absence of buffers produced similar lesions as seen in the previous experiments or, if not, the possible interaction of buffers in ammonia toxicity.

EXPERIMENTS WITH CARP

7.2 MARETIALS AND METHODS

Common carp weighing 8.76 ± 2.5 (±SD) grams and measuring 8.30 ± 0.9 (±SD) cms were used in these tests. Only one experimental trial was conducted with a duplicate set of tanks being used for each of the three ammonia concentrations tested with one duplicate set of controls. The experimental system, set up, and the number of fish per tank and the acclimation procedures remained similar to those in the previous experiments. The sampling procedure was also identical with five fish being sampled from each tank after three days of exposure and the remaining five fish after seven days exposure.

A dilution water of hardness 50.0 ± 1.65 (± SD) mgl^{-1} as $CaCO_3$ was used with no added buffers. The initial pH of this dilution water varied between 8.01 to 8.14 and the temperature adopted was around 27°C. Water quality parameters were monitored throughout the experimental period for each individual tank, on between three and five occasions and the mean values were

calculated.

7.3 RESULTS

7.3.1 WATER QUALITY

The results of the water quality parameters measured during the experimental period are summarized in Table 7.1. Although the initial pH of the dilution water measured was around pH 8.09, over the experimental period variations within the measured pH values of the individual tanks were noted. Such variations remained the same between the treatments as could be seen from means of the mean values for each duplicate set of tanks (mean ± SE) (table 7.1).The overall variations encountered ranged between pH 7.69 and pH 8.09 (minimum and maximum values measured over the experimental period).

Thus at the highest concentration of ammonia tested in the present experiments, the unionized ammonia concentrations would have fluctuated between 1.02 - 2.39 mgl^{-1}, with an overall mean value of 1.66 mgl^{-1} UIA. Apart from pH, the other water quality parameters did not fluctuate very much and were relatively constant. Also in the present experiment, as a result of the absence of any buffer, the electrolyte conductivity of the test solutions measured were very low in comparison with those of the previous experiments where buffers were employed. The smaller differences noted in the measured electrolyte conductivity in the present experiment were as a result of the presence of varying amounts of ammonium chloride. The nitrite-nitrogen values recorded remained insignificantly low and far below the proposed safe nitrite-nitrogen criteria for coarse fish (EIFAC 1984).

Table 7.1 Physico-chemical characteristics of the test solutions measured during the carp experimental period (trial 1)*1, mean ± SE, n = 2

	NH_4Cl mgl^{-1}	Total Ammonia-N mgl^{-1} Mean ± SE	NH_3-N*2 mgl^{-1}	Temperature °C Mean ± SE	pH Mean ± SE	Dissolved oxygen mgl^{-1} Mean ± SE	Total Alkalinity $meql^{-1}$	Electrolyte Conductivity $nS\ cm^{-1}$ Mean ± SE	Nitrite-N (NO_2-N) mgl^{-1} Mean ± SE
Control	0.00	2.943 ± 0.347	0.168	26.6 ± 0.06	7.98 ± 0.02	8.6 ± 0.05	1.15 ± 0.04	346.65 ± 3.35	0.015 ± 0.002
1	62.59	15.903 ± 0.227	0.879	27.08 ± 0.12	7.95 ± 0.02	8.15 ± 0.40	1.36 ± 0.35	415.0 ± 1.67	0.018 ± 0.002
2	95.00	24.319 ± 0.139	1.179	27.4 ± 0.00	7.88 ± 0.01	7.83 ± 0.33	1.53 ± 0.27	445.0 ± 3.30	0.005 ± 0.0002
3	127.99	31.86 ± 0.33	1.66	27.15 ± 0.13	7.92 ± 0.03	7.95 ± 0.41	1.42 ± 0.40	482 ± 7.5	0.010 ± 0.001

*1 The total hardness of the dilution water used in this trial was 56.0 (SD, 1.65, n=8) mgl^{-1} as $CaCO_3$.

*2 NH_3-N concentrations were calculated from the measured mean values of total ammonia, pH and temperature.

7.3.2 MORTALITIES AND BEHAVIOURAL RESPONSES

No mortalities were noted in any of the experimental treatments during the experimental period. Carp exposed to 0.88 and 1.18 mgl^{-1} unionized ammonia showed relatively minor signs of distress during the first two to three days, after which they appeared relatively normal. However, at 1.66 mgl^{-1} unionized ammonia fish exhibited severe neurological signs, with the loss of equilibrium and occasional hyperexcitability. Such behavioural signs continued for the first three to four days after which on the fish became relatively less distressed except for the loss of equilibrium exhibited by some fish.

7.3.3 HISTOPATHOLOGY

CONTROL FISH

The control carp showed no tissue or cellular alterations in the gills, both after three and seven days of exposure (Plate 60).

FISH AT 0.88 mgl^{-1} UIA

At low concentrations of ammonia a moderate increase in the concentration of chloride cells in the interlamellar regions and an increased appearance of such swollen chloride cells on the lamellar epithelium was seen.

An increased infiltration of eosinophilic granular cells into the gill epithelium and connective tissues was also clearly evident. Increased mucus secretion was once again a characteristic feature at low levels of ammonia with no obvious necrotic changes and vacuolation of mucous cells. A generalized loosening of the squamous epithelial cell layer of the filament

and lamellae was often noted in some areas. Cytoplasmic vacuolations were particularly visible in the epithelial layer of cells within the interlamellar regions. Nodular epithelial swellings were very rare but when found occasionally they were small, comprising of only a fewer degenerating cells. Pyknotic cells were occasionally seen amongst the normal looking cells of the epithelium.

At 1.18mgl^{-1} UIA

At this higher concentrations of ammonia smaller nodular swellings could be occasionally seen, but they were neither seen in large number nor did they increase in their size to any great extent unlike those of the phosphate or tris-buffered experiments. However, sloughing of lamellar and filamental squamous epithelium was more common at this concentration of ammonia. The sloughing of mucus and mucous cells along with the epithelial cells was more extensive. The densities of EGCs also greatly increased in the epithelia and the connective tissues. Extensive degranulation and degeneration of the EGCs was noted in the connective tissues and the gill epithelium. Frequently EGCs were also seen within the secondary lamellae of these small fish. Mucous cell exhaustion and vacuolations were also noted in the gill epithelia. In addition to these changes, the degeneration of the muscle bundles that support the gill rods (the cartilaginous gill rods of the filaments) was extensive.

At 1.66 mgl^{-1} UIA

At the highest concentration of ammonia (1.66 mgl^{-1} unionized ammonia) the lesions were almost identical to these noted at

1.18 mgl^{-1} UIA, but the intensity of the necrotic changes had increased. More pyknotic cells appeared within the epithelia and generalized cellular necrosis and vacuolations were much more obvious. The nodular lesions were once again fewer and smaller in size in comparison with those of the carp exposed to similar ammonia concentrations in phosphate or tris-buffered experiments (Plate 61b and 62). Chloride cell swelling and necrosis on the lamellar epithelium was more commonly seen (Plate 61a). Loosening of the outer squamous epithelial layer with increased sloughing, along with an increase in the cytoplasmic vacuolations were more characteristic changes seen in these experiments (Plate 63) when compared with those of the buffered experimental fish (Chapters 4 & 6) where the nodular swellings were more dominant.

In the combined AB and PAS staining at pH 2.5, there was an increase in acidic mucous cells staining strongly AB positive, and these cells were augmented with a second type of cell that was more green in staining and extended from the deeper layers into the surface region. Extenwsive sloughing of the mucous was also seen (Plate 64).

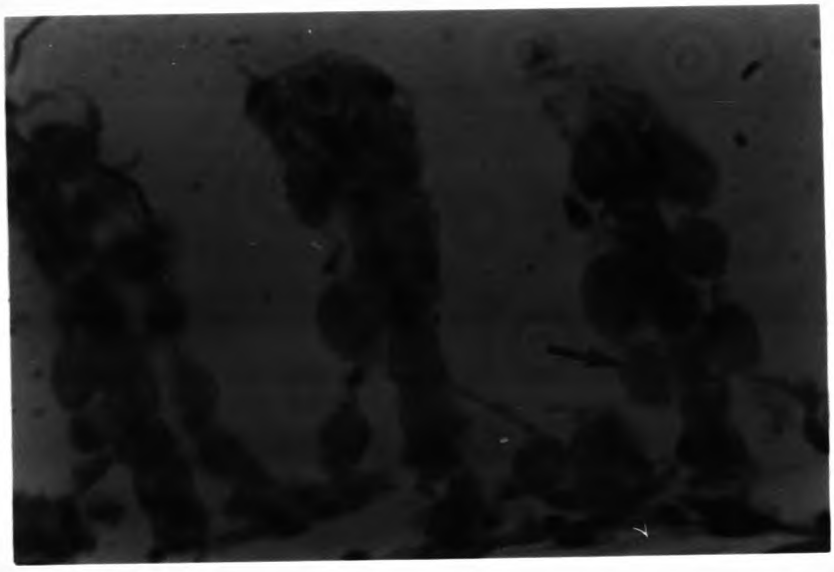
Plate 60.

Photomicrograph of control carp gills after seven days experimentation in unbuffered dilution water. H&E 1500X.

Plate 61.

a. Photomicrograph of carp gill lamellae from fish exposed to 1.18 mgl^{-1} UIA, showing severe chloride cell proliferation and inclusions within the chloride cells (arrowed). H&E, 1500X.

b. Photomicrograph of the gill tissue across the filaments of carp exposed to 1.66 mgl^{-1} UIA for seven days showing the nodular epithelial lesions in the interfilamental region (arrowed). Also present are PAS positive eosinophilic granular cells. PAS, 375X.



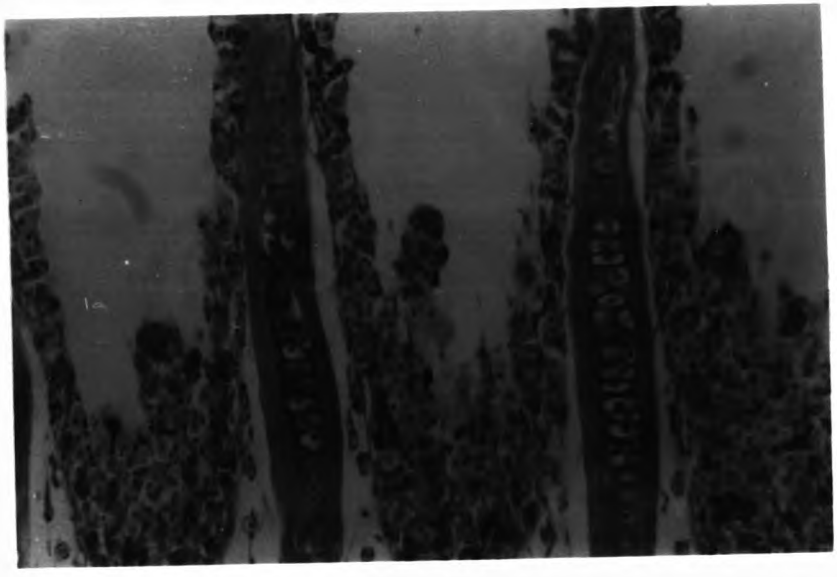


Plate 62.

a. Photomicrograph of the nodular lesions on the gill arch epithelium (arrowed) of carp exposed to 1.66 mgl^{-1} UIA for seven days.PAS 600X.

b.Changes in the mucous cells of the gill epithelium at 1.66 mgl^{-1} UIA, showing the occlusion of the mucous cells (arrowed)and vacuolations. PAS 600X.

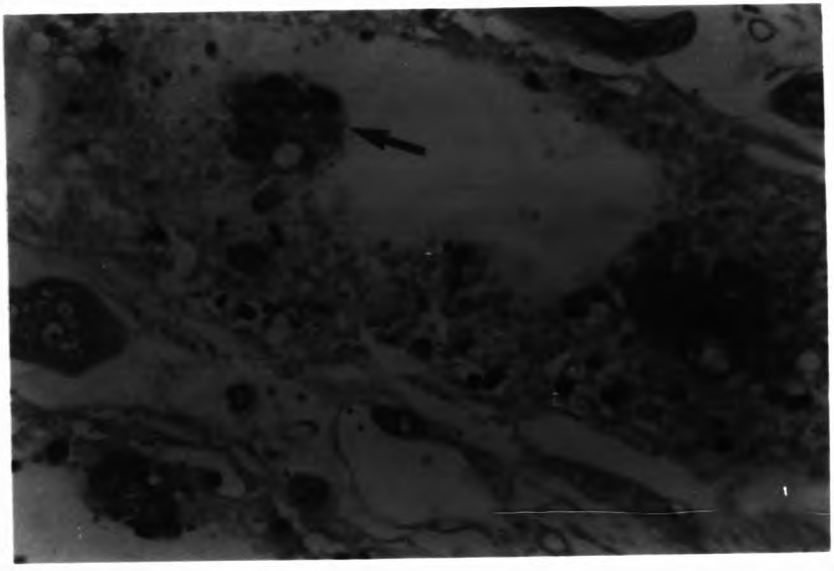
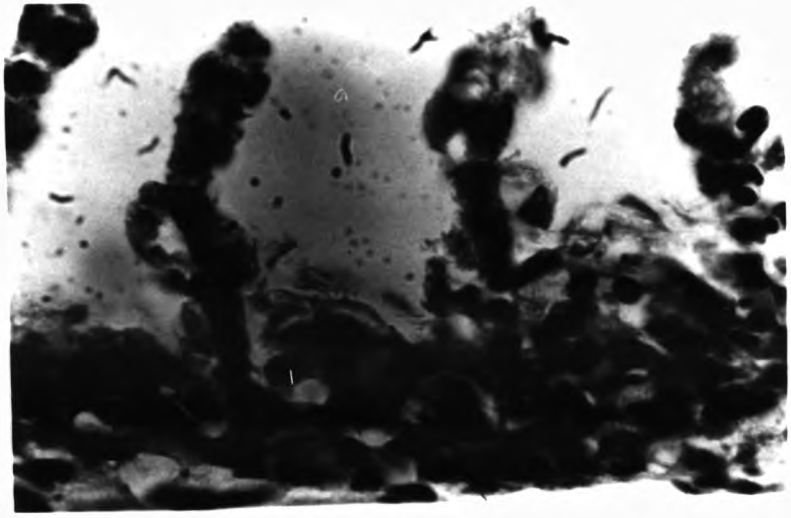


Plate 63.

Photomicrographs of the carp lamellae showing loosening of the squamous epithelium (a) and progressive sloughing at the ammonia concentrations of 1.66 mgl^{-1} UIA after seven days exposure.

H&E,1500X.



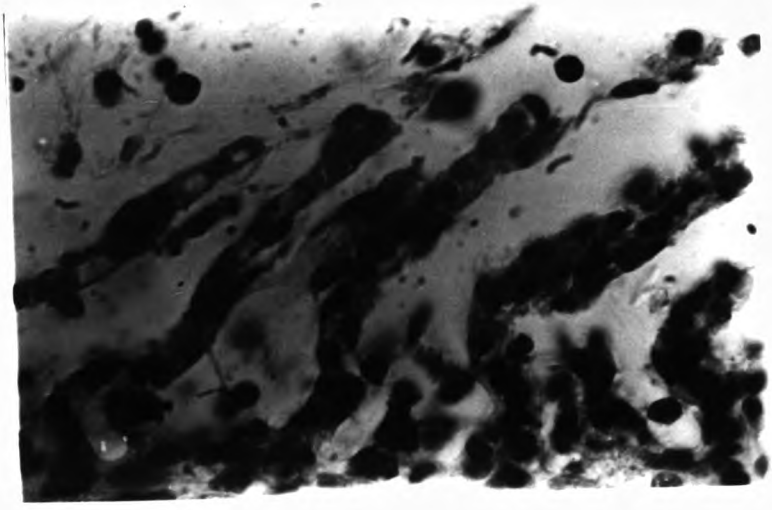
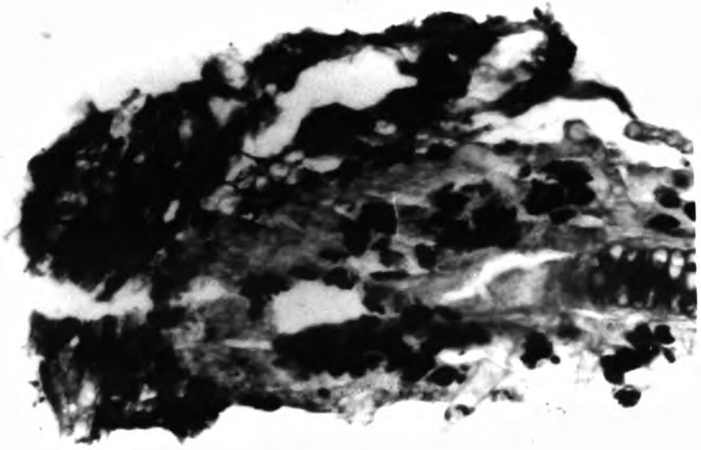


Plate 64.

Photomicrographs the carp gill tissues showing profuse mucous secretions at 0.88 mgl^{-1} UIA after three days exposure;

(a) on the tip of the gill filament and lamellae PAS,600X.

(b) on the gill rakers PAS,600X.



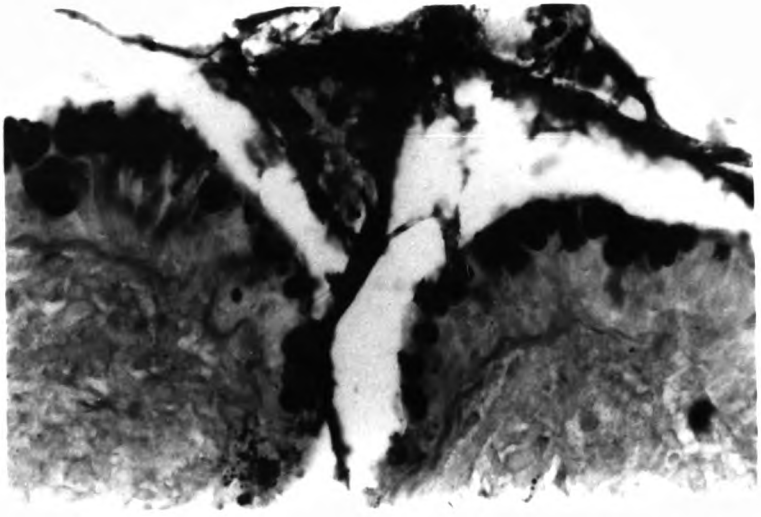


Plate 65.

Necrosis and vacuolation of mucous cells (small arrows) and eosinophilic granular cells (big arrows) within the filament epithelium of carp exposed to 1.66 mgl^{-1} UIA, for seven days. PAS,1500X.

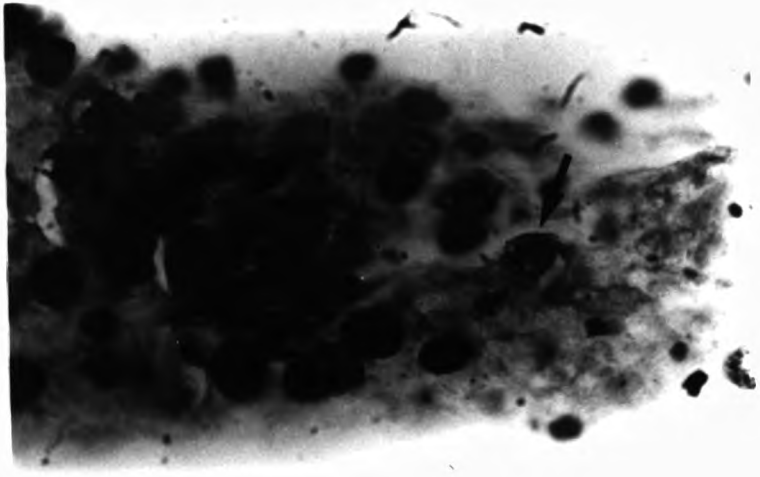
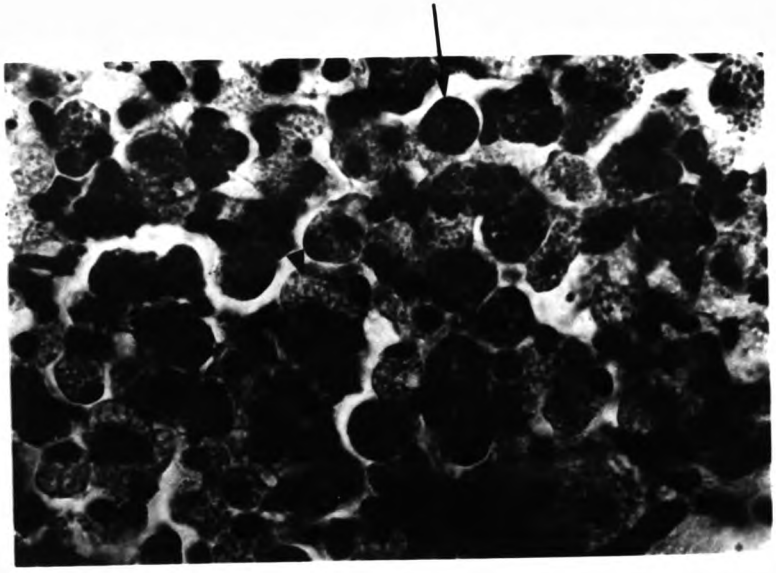


Plate 66.

Photomicrograph showing intense infiltration of eosinophilic granule cells in the connective tissues of the gill septum in carp exposed to 1.66 mgl^{-1} UIA for three days. Note the presence of both dark staining intact EGCs (arrowed) and pale staining degranulated EGCs (arrow heads). PAS,1500X.



7.4 DISCUSSION

The current experimental results indicate that the carp gill lesions due to ammonia in non buffered water varied from those seen in buffered experiments in two aspects. Firstly the occurrence of epithelial nodular swellings was relatively minor and secondly the loosening of the epithelial surface layers and their subsequent sloughing was increased greatly. However, the other characteristic changes such as: (1) chloride cell proliferation, swelling, and subsequent necrosis, 2) infiltration of EGCs into the gill tissues their degranulation and degeneration (Plate 66) and (3) mucus and mucous cell sloughing and vacuolations, were common in this experiment (Plates 64,65) .

A considerable reduction in the occurrence of nodular swelling in the gills of carp exposed to ammonia in non-buffered dilution water as compared with those of the previous buffered carp experimental results (Chapters 4 and 6), and a consequent increase in the loosening of the surface epithelium suggests that the carp gill lesions due to ammonia are being altered to some extent in the presence of buffers. It was also noted in the previous carp experiments that the epithelium was less affected generally, and most often the nodular swellings in the epithelium retained an intact outer squamous epithelial layer.Also the lamellar epithelium was less affected in terms of loosening or sloughing. However, in the present experiments, the nodular lesions were significantly fewer in and the ammonia appeared to be affecting more of the surface epithelium directly in contact with the water, along with subsequent cellular degeneration in the deeper layers.Thus it appears that the integrity of the cell and cellular membranes, particularly

those of the squamous epithelial layers, were protected in the presence of both buffer and ammonium chloride, while in the absence of buffer and presence of ammonium chloride alone there is no such protection.

Since the pH of the present non buffered experimental solutions remained relatively more alkaline than those of the buffered experiments where the pH was maintained within a narrow range, the unionized form of ammonia would have been obviously more predominant in the current experiments than those of the buffered experiments. Thus the predominance of more toxic unionized forms of ammonia at the gill surface might have been the cause of the changes seen such as the loosening of the squamous epithelial layer and subsequent sloughing (Plate 63,64).

An increase in the electrolyte concentration as noted by the very high values of electrolyte conductivity in the buffered experiments, which were also augmented by the use of relatively high amounts of ammonium chloride for the maintenance of required UIA concentrations, might have had a stabilizing effect on the cell membranes. On the other hand, the absence of such an effect in the non buffered experiments where the electrolyte concentrations were low and proportionately more irritant UIA was present, would have been in fact responsible for the actual differences in the histopathology noted. experiments.

The present experimental results are in more agreement with those of Flis (1968a,b) where the degenerative changes and sloughing of squamous epithelium and mucous and mucus cells was

predominant. Since Flis (1968a,b) did not use any added buffers in his experimental studies, but the alkaline pH was maintained rather by the use of ammonium hydroxide which served both as a buffer and a source of ammonia, the lesions described by Flis(1968a,b) do not mention the presence of any nodular epithelial lesions of the type noted in the previous phosphate or tris-buffered experiments, but rather more degenerative changes resulting in the loss of the epithelia, similar to the current experimental observations .

EXPERIMENTS WITH TROUT

7.5 MATERRIALS AND METHODS

Rainbow trout used in this trial had a mean weight of 17.98 ± 3.99 (±SD) grams and a mean length of 12.11 ± 0.91 (± SD) cms. A single experimental trial was conducted with two concentrations of ammonia and a control. Due to the shortage of available fish the treatments could not be replicated and hence only one test tank per concentration was used. The two concentrations of ammonia tested were 0.57 mgl^{-1} UIA and 0.76 mgl^{-1} UIA. The experimental procedure, set up, number of fish per tank and other procedures were similar to those of the previous experiments on trout, except that the water was not buffered.

RESULTS

WATER QUALITY

The means (±SD) of the water quality parameters measured for each of the experimental tanks during the experimental period are provided in Table 7.2. The values provided are the means of at least 4 estimations of each parameter made during the experimental period.

Table 7.2 Physico-chemical characteristics of the test solutions measured during the trout experimental period (trial 2)*1, mean ± SD, n = 4-6

	Total Ammonia mgl^{-1} Mean ± SD	NH_3-N*2 mgl^{-1}	Temperature °C Mean ± SD	pH Mean ± SD	Dissolved oxygen mgl^{-1} Mean ± SD	Alkalinity mgl^{-1} Mean ± SD	Electrolyte Conductivity nS cm^{-1} Mean ± SD	Nitrite-N (NO_2-N) mgl^{-1} Mean ± SD
Control	0.816 ± 0.233	0.014	15.0	7.79 ± 0.012	9.55 ± 0.05	0.62 ± 0.10	145.0 ± 7.07	0.0012 ± 0.0005
1	34.170 ± 0.960	0.570	15.0	7.80 ± 0.11	9.45 ± 0.05	0.71 ± 0.19	380.0 ± 176.82	0.002 ± 0.0008
2	43.632 ± 1.526	0.756	15.0	7.81 ± 0.12	9.55 ± 0.05	0.74 ± 0.20	433.33 ± 214.53	0.002 ± 0.0003

*1 The total hardness of the dilution water used in this trial was 27.75 (SD, 1.09, n=6) mgl^{-1} as $CaCO_3$.

*2 NH_3-N concentrations were calculated from the measured mean values of total ammonia, pH and temperature.

7.5 MORTALITIES AND BEHAVIOURAL CHANGES

No mortalities were noted in either of the ammonia concentrations in spite of their concentrations being higher than the earlier experiments. However, behavioural changes as described at the higher concentrations of ammonia in the previous experiments were noted at both the ammonia concentrations of the present experiment. Distressed neurological signs were once again more predominant at the highest ammonia concentration, where some fish were partially paralysed and lay at the bottom untill the end of the experiment.

7.6 HISTOPATHOLOGY

The control fish possessed normal gills with no noticeable changes (Plate 67). However, in the case of trout gills exposed to ammonia concentrations the typical changes as noted in the earlier experiments were present. Notable changes of ammonia exposure such as an increased chloride cell proliferation, oedematic changes in the epidermis, mucous cell vacuolation and necrosis were common. The loss of mucus was greater at both the ammonia concentrations (Plates 68,69,70). Pyknotic changes were noted both in the lamellae and the filament epithelium and were once again a clear indication of the toxic effect of ammonia on the gills.

Such epithelial loosening and sloughing as seen in the carp gills was not seen in trout (except in the case of mucus) and the lesions were similar to those seen in the previous buffered experiments (Chapters 5 & 6) at 25 ppm hardness. Thus the

histopathology of the trout gills in the current experiment was not altered in the absence of buffers and the pathological changes were similar.

7.7 DISCUSSION

The absence of any fish mortalities at a high concentration of ammonia in the present test (0.76 mgl^{-1} UIA), reemphasizes the general opinion that at higher pH unionized ammonia is less toxic (Redner *et al.* 1980; Thurston *et al.* 1981; Szumski *et al.* 1982; Sheehan and Lewis 1986). Although theoretically the ammonia toxicity has been known to increase positively with increasing pH, in fact it has been noted by the authors mentioned above that this is not actually the case. For example, the LC 50 values of UIA for rainbow trout at pH 6.80, 7.30, 7.82 and 7.87 were respectively 0.152, 0.374, 00.523 and 0.558 mgl^{-1} (Thurston *et al.* 1981). Similar results were noted by Sheehan and Lewis (1986) where the LC50 values of UIA for channel catfish at pH 6.0, 7.2, 8.0 and 8.8 were 0.74, 1.04, 1.45 and 1.91 mgl^{-1}. Thus these results indicate that the ammonia toxicity in terms of unionized ammonia is less toxic as the pH increases. Therefore the absence of mortalities noted here may be as a result of this effect.

On the other hand the pathology did not change in any way from that seen in the phosphate and tris-buffered experiments as detailed in Chapter, 5 and 6. Thus these experimental results confirm that the gill pathology in trout due to ammonia is not influenced by the presence of buffers within the time period of these experiments.

The experimental results noted in these different trials on

trout are in agreement with the general opinion that ammonia does causes such cellular changes such as lamellar hypertrophy (ie. chloride cell proliferation), enhanced mucus secretion, oedema and vacuolation (Smart, 1975; Smith and Piper, 1975; Thurston *et al.* 1984; Klontz *et al.* 1985), although changes such as telangiectasis and severe hyperplasia are questionable.

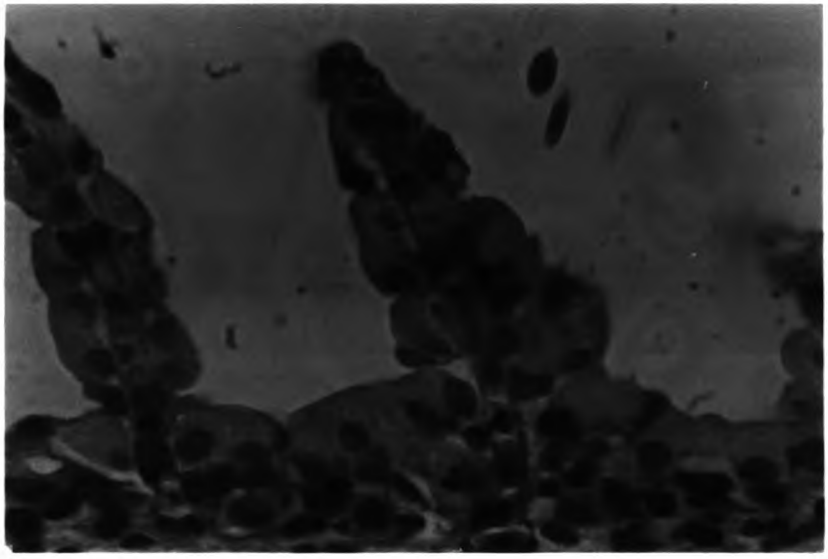
Plate 67.

SEM of control trout gills after seven days in non buffered dilution water showing normal gill structure. 200X.

Plate 68.

(a) A photomicrograph of trout gill lamellae exposed to 0.57 mgl^{-1} UIA for seven days showing severe chloride cell proliferation and hypertrophy. H&E, 1500X.

(b) Trout gill lamellae from fish exposed to 0.76 mgl^{-1} UIA for seven days showing severe chloride cell proliferation and cytoplasmic vacuolations. H&E, 1500.



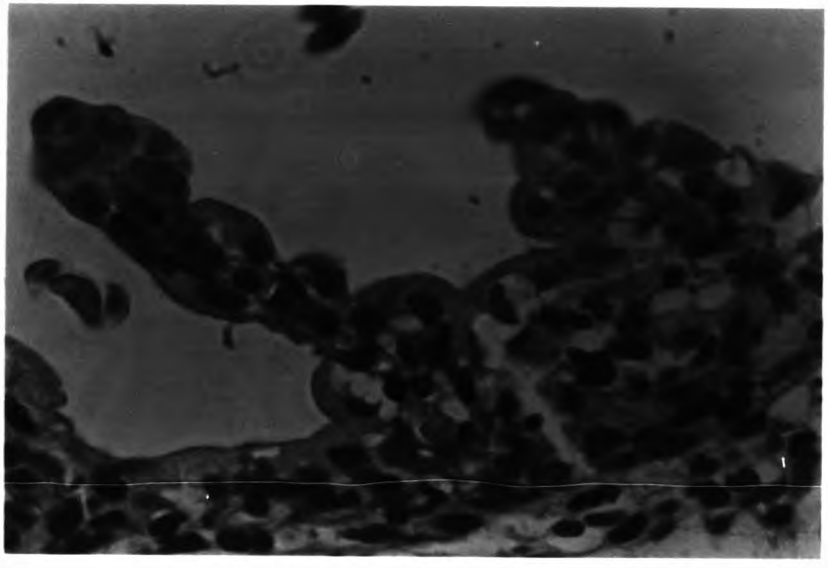
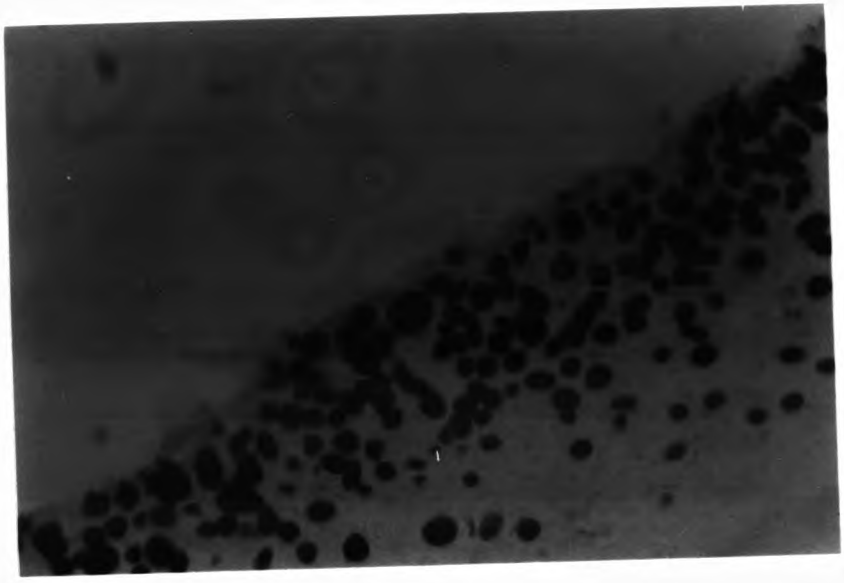


Plate 69.

a. A photomicrograph of gill arch epithelium showing normal distribution of mucous cells. PAS/AB at pH 2.5, 600X.

b. A photomicrograph of gill arch epithelium of trout exposed to 0.57 mgl^{-1} UIA for seven days showing mucous cells with flocculated contents and undergoing necrosis.PAS/AB pH 2.5, 600X.



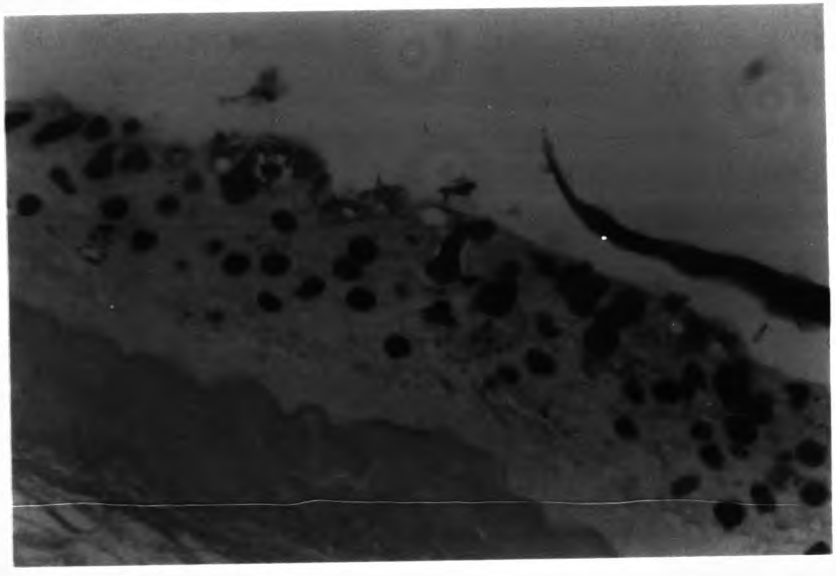
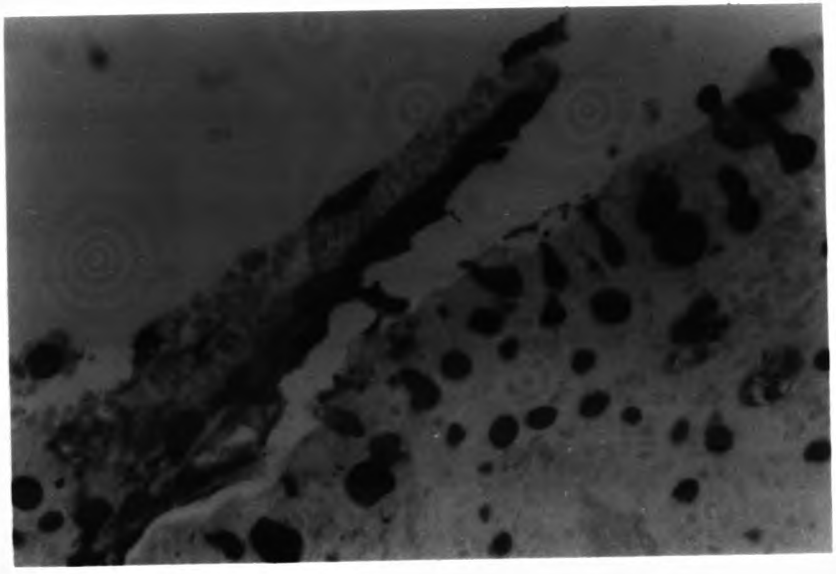
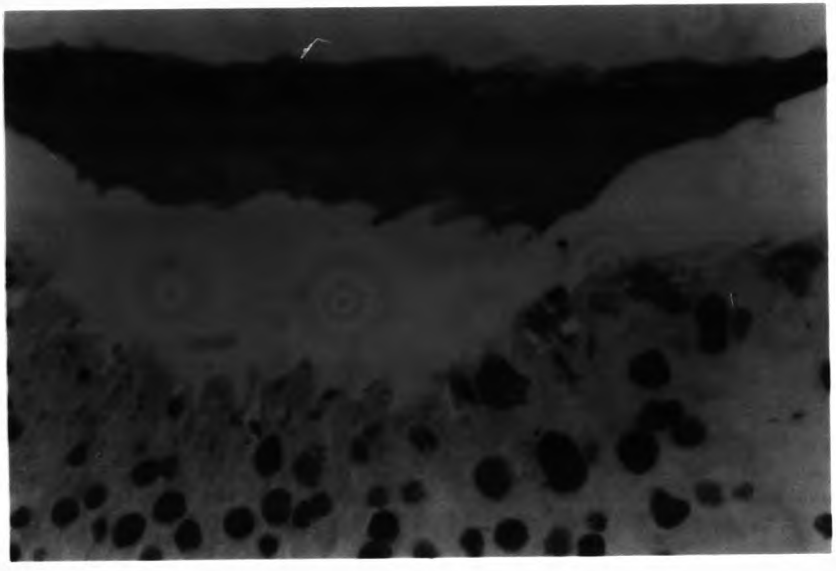


Plate 70.

Photomicrographs of gill arch epithelium from trout exposed to 0.76 mgl^{-1} UIA,for seven days. Note severe sloughing of the mucus and surface epithelium.PAS/AB at pH 2.5, 600X.





CHAPTER 8

SUMMARY AND GENERAL DISCUSSION

The effects of ammonia on the gill tissues of carp and trout were investigated in static experiments under standard water quality conditions, using recommended buffers for the maintenance of a constant pH, to provide a comparative gill pathology caused by ammonia.

A standard dilution water of 50ppm hardness was used in carp experiments, while in the case of trout a standard dilution water of 25ppm hardness was used in all but one experiment where a high hardness dilution water of 250ppm was also tested.

Phosphate and tris-buffers were used at concentrations generally adopted in the toxicity studies.

The phosphate buffer used was a combination of sodium dihydrogen phosphate and disodium hydrogen phosphate at 0.01M concentration,while the tris-buffer used was a combination of trizma base and trizma hydrochloride at 0.01M concentration.

Finally experiments were also conducted in dilution water only, with no buffers added.

Histopathological studies carried out included an extensive examination of light microscopic gill histological sections, augmented by both scanning and transmission electron microscopic studies.

Changes noted in the most common cell types of the gill epithelia covering the gill arch, filaments and lamellae were described.

From the experimental results presented in this thesis it is very clear that distinctive pathological changes attributable to toxic ammonia concentrations were recorded in both carp and trout gill tissues.

In the case of trout no apparent variations in pathology due to the toxicant were noted under three different water quality conditions tested (namely, phosphate buffered dilution water of 25ppm hardness, tris-buffered dilution water of 25ppm hardness,and unbuffered dilution water of 25ppm hardness), except for the more severe behavioural and neurological signs in the tris-buffered experiments.The major disadvantage of tris-buffer was its interference with the ammonia in the water and occasional precipitations.

The pathological changes noted in the trout gills included an enhanced chloride cell proliferation and necrosis, an increased infiltration of the EGCs and their degranulation, and an increased mucus secretion, mucous cell exhaustion, hypertrophy and vacuolation. Cellular oedema was one of the most characteristic changes in these experiments apart from the above mentioned changes.

However, at the high hardness level, in tris-buffered trout experiments the pathological changes were more severe and pronounced. Profuse mucus secretions were noted in the early stages, but towards the end of the experiments severe vacuolations and total exhaustion of a majority of the mucous cells occurred all over the gill epithelium. A generalized cellular condensation and necrosis of the cells of the gill epithelium was noted. Nuclear changes characterised by changes

such as pyknosis and karyorhexis were also increased. Severe hypertrophy of the cells of the lamellar epithelium and vacuolations were, however, the other most conspicuous changes under high hardness conditions.

Thus these experiments on trout confirm that ammonia brings about changes such as lamellar hypertrophy (which could be more appropriately refered as lamellar chloride cell proliferation and hypertrophy), increased mucus secretion and mucous cell necrosis, increased infiltration of EGCs, cellular vacuolations and oedema, and changes such as pyknosis and karyorhexis.

These experiments have also demonstrated that the pathology due to ammonia was not apparently influenced by the buffers used when such results were compared with nonbuffered experiments under identical water hardness conditions. However in tris-buffered experiments ammonia appears to be more toxic and under the conditions of high hardness the pathological changes noted in the gills are more severe and detrimental.

Hardness has been known to have no significant effect on the toxic effect levels of ammonia on fish (Emerson *et al.*, 1975; Alabaster and Lloyd 1980), but there is no experimental evidence available as to the effects of ammonia on fish tissues under high hardness conditions. Subjecting fish reared under low water hardness conditions to a very high hardness water will have an obvious osmotic impact due to an increased ionic concentration at the gill surface. Although fish such as rainbow trout have the innate capacity to adjust to such conditions an initial increase in the gill metabolic

activity is inevitable. Since ammonia in itself under low hardness conditions can bring about such changes as increased chloride cell proliferation and hypertrophy, apart from other changes that have been described, the more pronounced changes noted under high hardness and high ammonia conditions thus appear to be the combined effect of increased hardness and ammonia.

In the case of carp, entirely different and peculiar observations were noted in addition to some of the changes already noted in ammonia exposed trout.

Firstly the carp gills reacted in a very drastic way to the phosphate buffer, resulting in severe and extensive hyperplasia and hypertrophy of the filamental as well as lamellar epithelium. Intense mucous cell proliferation and the appearance of a type of specialized cells so far undescribed were noted in the inter lamellar and lamellar epithelia of the control fish. Also, an enhanced infiltration of the EGCs and other cells of probable leucocytic origin occurred.

Such drastic changes were surprisingly absent in similar buffered treatments that contained various amounts of ammonia in the form of ammonium chloride. At the lowest level of ammonia concentration tested (0.5 mgl^{-1} UIA,= 18 mgl^{-1} TA,= 68 mgl^{-1} NH_4Cl), no tissue damage was noted. While at 1.0 mgl^{-1} UIA,(= 36 mgl^{-1} TA,=131 mgl^{-1} NH_4Cl) only mild changes representing a chloride cell proliferation, infiltration of EGCs and a different type of lesion taking the form of nodular epithelial swelling was noted at low intensity. But at higher ammonia concentrations of 1.5 mgl^{-1} (=53 mgl^{-1} TA,=197 mgl^{-1}

NH_4Cl) the nodular lesions were quite extensive and were noted all over the gill epithelium except the lamellar epithelium.The infiltration and degranulation of the EGCs was also extensive and the epithelium in general appeared more granular or vacuolar as a consequence of necrotic changes in the EGCs. Changes in the mucous cells and epithelial cells were also conspicuous.

When these experiments were repeated with tris-buffered dilution water the control fish did not develop any pathology or tissue alterations but remained normal. However in fish exposed to ammonia, mild lesions of the type noted in the phosphate buffered experiments were noted at concentrations of ammonia as low as 0.42 mgl^{-1} UIA (=20.0 mgl^{-1} TA, = 76.4 mgl^{-1} NH_4Cl) and 0.61 mgl^{-1} UIA (=16.90 mgl^{-1} TA,= 64.5 mgl^{-1} NH_4Cl), while the lesions at higher concentrations of ammonia were extensive and identical to those seen in the case of high ammonia exposed phosphate buffered experiments.

Finally when these experiments were repeated in unbuffered dilution water a variation in the type of tissue lesions was noted. The extensive nodular tissue lesions as seen in the case of high ammonia exposed carp in phosphate buffered and tris-buffered waters were seen only on a minor scale, even at ammonia concentrations as high as 1.66 mgl^{-1} UIA (= 31.86 mgl^{-1} TA,=127.99 mgl^{-1} NH_4Cl). Instead filamental and lamellar epithelial loosening and sloughing was predominant. Changes in the EGCs, chloride, cells and mucous cells were as in the other experiments.

Thus these results on carp in particular have demonstrated

that although the pathological manifestations due to ammonia in carp are more regressive and necrotic in comparison with those of trout,nevertheless they more variable depending on the water quality and the chemical substances used in buffering the water.

In trout, although such variations in pathology as seen in the carp gills under identical water hardness were not seen, nonetheless an extreme variation in the pathology was noted at high hardness and high ammonia conditions. Thus the overall results from the carp and trout experiments suggest that variations in the water quality are primarily responsible as a cause of variability in the pathology due to the toxicant. In other words these results suggest that the possible wide variations that have been reported in the literature both on the histopathology and lethal level values of ammonia to fish might be accounted for by such discrepancies in water quality resulting from the variations in the hardness, alkalinity, conductivity and use of chemical salts to regulate the pH.

For instance the median tolerance limits of ammonia reported by Rice and Stokes(1975) for rainbow trout wherein tris-buffer was used at 0.05M to maintain the pH, was only 0.072 mgl^{-1} as UIA for both fry and adult fish. This value is not only at variance with the reported lethal threshold concentrations of ammonia to trout by other workers (Alabaster and Lloyd 1980; Meade 1985) but is ridiculously low. Such a mistake now appears to be due to the use of tris-buffer and the failure of the authors to visualize the interference of tris with ammonia measurement,rather than the fish being so

sensitive to ammonia as these author's results might suggest.

It can also be noted from the studies of Hasan and Macintosh (1986) that the LC50 values reported for carp fry of 0.2-0.3 gram size, in phosphate buffered dilution water are the highest so far reported for common carp of any size (Rao *et al.*1975; Dabrowska and Sikora 1986).Such higher values might have resulted as a consequence of the use of phosphate buffer.

However the 96 hour and 168 hour LC50 values reported by Hasan and Macintosh (1986) for young carp of only 0.2-0.3 mg size (at 1.74- 1.84 mgl^{-1} as unionised ammonia respectively) are by far the highest recorded in the literature. These values are approximately twice as much as those of the LC50 values reported by Rao *et al.*(1975) for common carp of 1-2 gram size (at 0.96 mgl^{-1} as UIA)and are considerably higher than those LC50 values of Dabrowska and Sikora (1986)for common carp of 23- 43 grams (48-h LC50 values being within 0.91-1.56 mgl^{-1} as UIA). Therefore it seems more probable that the phosphate buffer might have had some possibly a beneficial effect in the presence of ammonia. Further more Hasan and Macintosh(1986) reported some mortalities in the controls but not at low concentrations of ammonia,indicating that the phosphate buffer was probably detrimental in the absence of ammonium salts but became more toxic as the ammonia concentrations increased. Although the behaviour of the binary mixtures of the buffer chemicals and the ammonium salts is not well known it might be that the an enhanced Na concentration had some beneficial role by being available along with the Cl^- ion for the exchange processes. Redner *et al.* (1980) noted an alleviation of the short term ammonia toxicity due to the addition of sodium

chloride to the experimental medium in the case of channel catfish. These authors, while commenting that the sodium depletion was probably the contributing factor for the ammonia toxicity mechanism,later observed that such a protective mechanism did not last for more than 24 hours, as there was no significant difference in the LC50 values of NaCl supplemented and non supplemented experiments and concluded that there may be other possible ways of ammonia toxicity. However these results reflect a possibility that the fish may become more acclimatised during this short period of time and thus may in fact have an influence on the long term LC50 values, positively resulting in elevated values as noted by Hasan and Macintosh (1986).

Thus these results indicate that while the buffers themselves could be detrimental to fish, their effects in the presence of ammonia appears to be either antagonistic or additive, or both, depending upon the concentration of the toxicant ammonia and the buffer type involved.

In spite of the above disparities the most important outcome of these experiments is the finding that ammonia is extremely toxic to gill tissues and can cause necrotic and degenerative changes in various cell types and in particular the cellular components of the most active gill cells such as the chloride cells, the mucous cells and the eosinophilic granular cells.

The significance of such cellular damage of the gill tissue may only be understood with a due consideration of the structural and functional complexity of the gill tissue. From of the vast body of recent information that has been generated on the

ultrastructural and physiological aspects of the normal gill tissues, the branchial epithelium has been recognised as an extremely complex tissue in both its structure and physiological functions (Mommsen,1984). The fish gills constitute the major organs of respiratory gas exchange, play important roles for ionic and osmoregulatory balance, and are the main location for the excretion of the nitrogenous substances. In addition they also constitute the major site of diffusional exchange of water across the gills. All these functions, with the exception of the exchange of water, oxygen and carbon dioxide which seem to be due to diffusion are known to require a metabolic activity that is on a gram basis and in a resting animal surpasses all the remaining fish tissues. The high metabolic activity is also due the abundance of mitochondria in specialised gill cells such as chloride cells and mucous cells with defined functions.The musculature of the gill filaments and the pillar cells of the lamellae with their contractile properties are known to be obvious oxygen demanding components of the gills.In view of such an high metabolic stature,the effects of ammonia loading could be detrimental to physiological functioning of the gill tissue.Available information on the toxic effects of ammonia on the physiological processes indicates that ammonia has both a membrane and metabolic effect (Campbell, 1973; Heisler,1984; Randall and wright 1987). It is known to affect various membranal exchange processes including the mitochondrial K^+ and H^+ ion exchanges, thus affecting the oxidative processes of mitochondria (Campbell, 1973).

Stimulation of glycolysis, suppression of the tricarboxylic acid cycle and depletion of the cerebral energy metabolism have

been well documented in fish (Sousa and Meade 1977; Smart,1978; Chetty, Naidu, Reddy, Aruna and Swami 1980; Arillo *et al.*1981). Sousa and Meade(1977), also suggested that an accumulation of metabolic acids resulting from enzymatic stimulation of glycolysis by ammonia and suppression of the tricarboxylic acid cycle may cause oxygen to be prematurely released from haemoglobin by disturbing the blood buffer system.

According to Heisler (1984), during stress conditions the capacity of the excretory organs is usually not enough to prevent transient acid base disturbances as a result of continuous production of H^+ and OH^- ions which are ordinarily eliminated by the excretory organs under normal steady state condition. The buffering capacity of the fish blood and intracellular compartments are generally much smaller than in higher vertebrates, and that the fish's capacity for adjustment by increased respiratory compensation is also limited due to the relatively low oxygen content of the water and to the physical limitations and energetic problems with longterm hyperventilation of the viscous gas exchange medium. Thus these disturbances may be of greater significance in fish than in higher vertebrates. It has also been shown that the intensity of the exchanges of $Na^+/NH4^+$, $Na^+/H^+$and Cl^-/HCO_3^- is closely linked to the regulation of the internal acid-base equilibrium and the elimination of ammonium (Payan 1980). Thus an increased ammonia loading may be upsetting the ionic exchange processes as such due to its influence on acid-base regulation.

Since the maintenance of a constant pH in the body fluids at a given temperature is one of the important homeostatic tasks of regulatory systems in animals, disturbances in acid base regulation and resultant pH alterations may affect the enzyme

systems that catalyse the metabolic reactions which possess pH optima within a narrow range (Heisler,1984). Therefore the pathological processes noted in the gill tissues of high ammonia exposed fish, such as increased chloride cell proliferation and their necrosis, appears to represent an initial increased metabolic state and a progressive loss of control of cellular activity. The necrotic changes representative of the chloride cell mitochondrial swelling,loss of mitochondrial matrix, and appearance of dark inclusions within the chloride cells (carp gill particularly) seem to suggest a metabolic failure of these cells as a consequence of ammonia induced changes. These observations are identical to those described by Chow and Pond (1972) who have also reported mitochondrial swelling & loss of mitochondrial matrix along with the suppression of the citric acid cycle.

Apoptotic changes noted in the gill epithelium representing various stages of cellular and nuclear condensation and the phagocytosis of such cell by the adjacent cells suggests wider metabolic and membrane effects of ammonia in the gill tissues. Changes such as pyknosis, karyolysis, and karyorhexis as noted in this study have also been reported by various workers (Burkhalter and Kaya 1977; Calamari *et al.* 1981). Although excessive mucus secretions and mucous cell activity have been described as a result of ammonia exposure (Flis,1968b; Smart,1976 ; Calamari *et al.* 1981), relatively very little is documented on the structural changes of these cells. Lang *et al.* (1987) reported no evidence of ammonia having an influence on mucus production based on mucous cell counts of ammonia exposed trout. However their results are contradictory to the present observations and might have been

due to the loss or total exhaustion of the mucous cells.

As far as the eosinophilic granular cells are concerned, these cells appear to be playing a possible defence role and further investigations may be necessary to understand their precise role. The inter and intra cellular oedema characteristically seen in trout gills may be due to increased permeability of the gill epithelium as has been suggested (Lloyd and Orr 1969; Thurston *et al.* 1984). However, such oedema was not conspicuous in carp but instead the cellular vacuolations were characterised by the vacuolated or degranulated EGCs. The appearance of the nodular hyperplastic epithelial swellings in carp appear to be species specific, been reported in fathead minnows (Smith, 1984) where the hyperplastic lesions induced by ammonia were only seen in the primitive meninx surrounding the brain tissue. Smith (1984) also noted an abundance of rodlet cells amongst the hyperplastic tissue lesions, and such an increased occurrence was also noticed in the case of ammonia exposed carp although they were present in low numbers in the control gills. The role of these cells also needs to be investigated.

REFERENCES

Alabaster,J.S. & Herbert,D.W.M. (1954). Influence of carbon dioxide on the
toxicity of ammonia. Nature, London. 174,404-405.

Alabaster, J.S., Shurben, D.G. & Knowles, G. (1979). The effects of dissolved oxygen and salinity on the toxicity of ammonia to smolts of salmon, Salmo salar L. J. Fish Biol.15: 705-712.

Alabaster, J.S. & Lloyd, R. (1980). Ammonia ; In Water Quality Criteria for Fresh Water Fish. Butterworths, London, England. pp.180-102.

Alderson, R.(1979). The effects of ammonia on the growth ofjuvenile dover sole, Solea solea (L.) and turbot, Scophthalmus maximus. Aquaculture, 17, 291-309.

Al-Hussaini, A.H. (1949). On the functional morphology of the alimentary tract of some fish in relation to differences in their feeding habits: cytology & physiology. Quart. J. Micro. Sci. 90, 323-355.

APHA. (1980). Standard Methods for the Examination of Water and Waste water. 15th edition. Washington, D.C. American Public Health Association, pp1134.

APHA (1985). Standards Methods for the Examination of Water and Waste Water. 16th edition. Washington, D.C. American Public Health Association. pp1134.

Arillo, A., Margiocco, C., Melodia, F., Mensi, P. & Schenone, G. (1981). Ammonia toxicity mechanism in fish:studies on rainbow trout(Salmo gairdneri Rich).Ecotoxicol.Environ. Saf. 5,316-328.

Armstrong, D.A., Chippendale, D., Knight, A.W. & Colt, J.E. (1978). Interaction of ionized and unionized ammonia on short-term survival and growth of prawn larvae, Macrobrachium rosenbergii. Biol. Bull. 154, 15-31.

Bannister, L.H. (1966). Is Rhabdospora thelohani (Laguesse) a sporozoan parasite or a tissue cell of lower vertebrates? Parasitology. 56, 633, 638.

Blackstock, N & Pickering, A.D. (1980). Acidophilic granular cells in the epidermis of the brown trout, Salmo trutta L. Cell Tissue Res. 210, 359-369.

Bolton, L.L. (1933). Basophil (mast) cells in the alimentary canal of salmonid fishes. J. Morph. 54, 549-582.

Brockway, D.R. (1950). Metabolic products and their effects. Prog. Fish-cult. 12, 127-129.

Brown, V.M. (1968). The calculations of the acute toxicity of mixtures of poisons to rainbow trout. Water Res. 2, 723-733.

Brown, V.M., Shurben, D.G. & Shaw, D.(1970).Studies on water quality and the absence of fish from some polluted English

rivers. Water Res. 70, 363-382.

Brownell, C. (1980). Water quality requirements for first feeding in marine fish larvae. I. Ammonia, nitrite, and nitrate. J. Exp. Mar. Biol. Ecol. 44, 269-283.

Bucke, D. (1971). The anatomy and histology of the alimentary tract of the carnivorous fish the pike (Esox lucius). J. Fish Biol. 3, 421-431.

Buckley, J.A. (1978). Acute toxicity of unionized ammonia to fingerling coho salmon. Prog. Fish-cult. 40, 30-32.

Burkhalter, D.E. & Kaya C.M. (1977). Effects of prolonged exposure to ammonia on fertilized eggs and sac fry of rainbow trout (Salmo gairdneri). Trans. Am. Fish. Soc. 106, 407-475.

Burrows, R.E. (1964). Effects of accumulated excretory products on hatchery reared salmonids. Res. Rep. U.S. Fish. Wildl. Serv. 66, 1-12.

Bullock, G.L. (1972). Studies on selected myxobacteria pathogenic for fishes and on bacterial gill disease in hatchery reared salmonids. U.S.Bur.Sport Fish. Wildl.Tech.Paper. 60,pp30.

Calamari, D., Marchetti, R. & Vailati, G. (1981). Effects of long-term exposure to ammonia on the developmental stages of rainbow trout (Salmo gairdneri Richardson). Rapp. P. -V. Reun. Int. Explor. Mer. 178, 81-86.

Caliborne, J.B., Evans, D.H. & Goldstein, L.(1982). Fish branchial Na^+/NH_4^+ exchange is via basolataral Na^+/K activated ATPase. J.Exp.Biol. 96,431-434.

Campbell,J.W.(1973).Nitrogen excretion. pp.279-316; In C.L. Prosser edited Comparative animal physiology. W.B.Saunders Company,Philadelphia.

Cameron, J.N. & Heisler, N. (1983). Studies of ammonia in the rainbow trout:Physiochemical parameters, acid-base behaviour and respiratory clearance. J. Exp. Biol. 105, 107-125.

Chow, K.W. & Pond, W.G. (1972). Biochemical and morphological swelling of mitochondria in ammonia toxicity. Proc. Soc. Exptl. Biol. Med. 139, 150-156.

Chetty, C.R., Naidu, R.L., Reddy, Y.S., Aruna, P. & Swami, K.S. (1980). Tolerance limits and detoxification mechanisms in the fish (Tilapia mossambica) subjected to ammonia toxicity. Indian. J. Fish. 27,177-182.

Colt, J. & Armstrong, G. (1981). Nitrogen toxicity to crustaceans, fish and mollusc. Proceedings of the bio-engineering symposium for fish culture, fish culture section. Am. Fish. Soc., (FCS) Publ. 1,34-47.

Cooper, A.J.L. & Plum, F. (1987). Biochemistry and physiology of brain ammonia. Physiol. Rev. 67, 440-519.

Crespo, S., Soriano, E., Sampera, C. & Balasch, J. (1981). Zinc and copper distribution in excretory organs of the dog fish scyliorhinus canicula and chloride cell response following treatment with zinc sulphate Mar. Biol. 65, 117-123.

Crespo, S. & Sala, R. (1986). Ultrastructural alterations of the dog fish (Scyliorhinus canicula) gill filament related to experimental aquatic zinc pollution. Dis.Aquat.Org. 1, 99-104.

Crespo, S., Padros, F., Sala, R. & Marlasca, M.J. (1988). Gill structure of cultured Salmo trutta fario related to sampling techniques. Dis. Aquat. Org. 4, 219-221.

Cruz, E.R. & Enriquez, G.L. (1982). Gill lesions associated with acute exposure to ammonia. Nat. Appl.Sci. Bull. 34, 1-13.

Cuthbert,A.W. & Maetz,J.(1972).Effects of Ca^{2+} and $Mg,^{2+}$ on sodium fluxes through the gills Carassius auratus. J.Physiol.(Lond). 221, 633-643.

Dabrowska, H. & Sikora, H. (1986). Acute toxicity of ammonia to common carp (Cyprinus Carpio L.). Polskie Archiwum Hydrobiologii. 33, 121-128.

Danecker, E. (1964). Die jauchevergiftung von fischen-eine ammoniakvergiftung. Österreiches Fischerei. 3/4, 55-68.

Daoust, P.Y. & Ferguson, H.W. (1984). The pathology of chronic ammonia toxicity in rainbow trout Salmo gairdneri Richardson.

J. Fish Dis. 7, 199-205.

Department of the Environment, U.K. (1972). Water Pollut. Res. 1971. London, H.M.S.O.

Desser, S.S. & Lester, R. (1975). An ultrastructural study of the enigmatic "rodlet cells" in the white sucker, Catastomus commersoni (Lacepede) (Pisces, Catastomidae). Can. J. Zool. 53, 1483-1494.

De Vooys, G.G.N. (1968). Formation and excretion of ammonia in teleostei. I. Excretion of ammonia through the gills. Arche int. Physiol. Biochim. 76, 268-272.

Doudoroff, P., Anderson, B.G., Burdick, G.E., Galtsoff, P.S., Hart, W.B., Patrick, R., Strong, E.R., Surber, E.W. & Horn, W.M.V. (1951). Bioassay methods for the evaluation of acute toxicity of industrial wastes to fish. Sew. Ind. Wastes. 23, 1380-1951.

Dougherty, W.J. (1981). Preparation of semithin sections of tissues embedded in water soluble methacrylate for light microscopy. In Staining procedures. 4th ed. (Clarck, G. ed). Williams and Wilkins, Baltimore. p.27-38.

Downing, K.M.& Merkens, J.C. (1955). The influence of dissolved-oxygen concentration on the toxicity of un-ionized ammonia to rainbow trout (Salmo gairdneri Richardson). Ann. Appl. Biol. 43, 234-246.

Driedzic, W.R. & Hochachka, P.W. (1976). Control of energy metabolism in fish white muscle. Am. J. Physiol. 230, 579-582.

Drury, R.A.B. & Wallington, E.A. (Eds). (1980). Carleton's Histological technique. Fifth edition, Oxford University Press. pp511.

EIFAC (European Inland Fisheries Advisory Committee). (1970). Water quality for european fresh water fish. Report on ammonia and inland fisheries. EIFAC Technical Paper No.37. Food and Agriculture Organisation of the United Nations. Rome, Italy.

EIFAC.(1984). EIFAC working party on water quality criteria for European fresh water fish. Report on Nitrite; Draft summary and conclusions and tentative water quality criteria. EIFAC/ X111/ 84/ 16/ sup.1.4

Eills,A.E.(1974).The eosinophil and mast cell of fishes - a review.PhD..thesis University of Aberdeen.

Emerson, K., Russo, R.C., Lund, R.E. & Thurston, R.V. (1975). Aqueous ammonia equilibrium calculations: Effects of pH and temperature. J. Fish. Res. Board. Can. 32, 2379-2383.

Evans, D.H. (1980). Kinetic studies of ion transport by fish gill epithelium. Am. J. Physiol. 238, R224-R230.

Evans,R.E.,Brown,S.B.& Hart,T.J.(1988).The effects of aluminum and acid on the gill morphology of rainbow trout Salmo gairdneri.Envion.Biol.Fish. 22, 299-311.

Flis, J.(1968a). Anatomicohistopathological changes induced in carp (Cyprinus carpio L.) by ammonia water. Part I. Effects of toxic concentrations. Acta. Hydrobiol. 10, 205-224.

Flis, J. (1968 b). Anatomicohistopathological changes induced in carp (Cyprinus carpio L.) by ammonia water. Part II. Effects of subtoxic concentrations. Acta Hudrobiol., 10 : 225-238.

Fisher, Z. (1977). Some remarks on nitrogen excretion by fish. Pol. Arch. Hydrobiol. 24, 355-360.

Fletcher, T.C. & White, A. (1973). Lysozyme activity in the plaice, Pleuronectes platessa L. Experientia 29, 1283-1285.

Flood, M.T., Nigrelli, R.F. & Gennaro, J.F. (1975). Some aspects of the ultrastructure of "Stabchendrusea-zellen", a peculiar cell associated with the endothelium of the bulbus arteriosus and with other fish tissues. J. Fish Biol. 7, 129-138.

Forster, R.P. & Goldstein, L. (1969). Formation of excretory products. In Fish Physiology, Vol.1 (Hoar, W.S. & Randall, D.J., eds), London and New York: Academic Press. pp313-350.

Fratantoni, J.C., Hall, C.W. & Neufeld, E.F. (1968). The defect in hurler's and hunter's syndromes: faulty degeneration of

mucopolysaccharide. Proc. Nat. Acad. Sci. (USA). 60, 699-706.

Fromm, R.O. (1963). Studies on renal and extrarenal excretion in a fresh water teleost. Salmo gairdneri. Comp. Biochem. Physiol. 10, 121-128.

Gerking, S.D. (1955). Endogenous nitrogen excretion of bluegill sunfish. Phys. Zool. 28, 283-289.

Girard, J.O. & Payan, P.(1980). Ion exchanges through respiratory and chloride cells in fresh water and sea water adapted teleosteans. Am. J. Physiol. 238, R260-R268.

Glauret, A.M. (1975). Practical methods in Electron microscopy. Volume 3, Part 1; Fixation ,dehydration and embedding of biological specimens. pp.201. North-Holland publishing Company.

Goldstein, L., Forster.,R.P. & Fanelli.G.M.Jr. (1964) Gill blood flow and ammonia excretion in the marine teleost.Myoxocaephalus scorpius. Comp.BIochem.Physiol. 21, 719-722.

Goldstein, L., Claiborne, J.B. & Evans, D.E. (1982). Ammonia excretion by the gills of two marine teleost fish: The importance of NH_4^+ permeance. J. Exp. Zool. 219, 395-397.

Golterman, H.L., Clymo, R.S. & Ohnstad, M.A.M. (1979). Methods for Physical and Chemical Analysis of fresh waters. IBP Handbook No.8, 2nd edition, Oxford, Blackwell. pp213.

Hampson, B.L. (1976). Ammonia concentration in relation to ammonia toxicity during a rainbow trout rearing experiment in a closed fresh water - sea water system. Aquaculture. 9, 61-70.

Harder, R.R. & Allen, G.H. (1983). Ammonia toxicity to chinook salmon parr: reduction in saline water. Trans. Am. Fish. Soc. 112, 834-837.

Harris, J.E. & Hunt, H. (1975). The fine structure of the epidermis of two species of salmonid fish, the atlantic salmon (Salmo salar L) and brown trout (Salmo trutta L.). II. Mucous cells. Cell Tiss. Res. 163, 535-543.

Harwood, J & Khun A.L. (1970). A colorimetric method for ammonia in natural waters. Wat. Res. 4, 805-811.

Hasan, M.R. (1986). Husbandry factors affecting survival and growth of carp *(Cyprinus carpio* L.) fry and an evaluation of dietary ingredients available in Bangladesh for the formulation of a carp diet. PhD, thesis, Institute of Aquaculture, University of Stirling, Scotland, UK. pp415.

Hasan, M.R. & Macintosh, D.J. (1986). Acute toxicity of ammonia to common carp fry. Aquaculture. 54, 97-107.

Hayat, M.A. (1978). Introduction to biological scanning electron microscopy. University park press. Baltimore, Maryland 21202. pp. 214.

Haywood, G.P. (1983). Ammonia toxicity in teleost fishes: A review. Canadian Technical Report of Fisheries and Aquatic Sciences No.1177. Department of Fisheries and Oceans. British Columbia. pp35.

Heisler,N.(1984).Acid-base regulation in fishes. in " Fish Physiology" (Hoar,W.S.& Randall,D.J.,eds),Vol.XA,pp315-401. Academic Press,New York.

Herbert, D.W.M. (1961). The toxicity to rainbow trout of spent still liquors from the distillation of coal. Ann. Appl. Biol. 50, 755-777.

Herbert, D.W.M. & Van Dyke, J.M. (1964). The toxicity to fish of mixtures of poisons. 2. Copper-ammonia and zinc-phenol mixtures. Ann. Appl. Biol. 53, 415-421.

Herbert, D.W.M. & Shurben, D.G. (1965). The susceptibility of salmonid fish to poisons under estuarine conditions. II. Ammonium chloride. Int. J. Air Wat. Pollut. 9, 89-91.

Hemens, J. (1966). The toxicity of ammonia solutions to the mosquito fish (Gambusia affinis Baird on Girard). J. Proc. Inst. Sew. Purif. 1966, 265-271.

Herman, R.L. & Meade, J.W. (1985). Gill lamellar dilations (Telangiectasis) related to sampling techniques. Trans. Am. Fish. Soc.114. 911-913.

Hoar, W.S. & Randall, D.J.(Eds). (1984). Terms and abbrevations: In Fish Physiology. Volume X,part A, pp Xiii.

Holden, A.V. & Lloyd, R. (1972). Symposium on the nature and extent of water pollution problems affecting inland fisheries in Europe. Synthesis of national reports. EIFAC Tech. Paper. No.16.

Hornich, M. & Tomanek, J. (1983). Necrotic processes in carp gill tissue caused by changes in aquatic medium. Vet. Med. (Poland). 28, 621-632.

Hughes, G.M. & Wright, D.E. (1970). A comparative study of the ultrastructure of the water-blood pathway in the secondary lamellae of teleost and elasmobranch fishes- benthic forms. Z. Zellforch. Mikrosk. Anat. 104, 478-493.

Hughes, G.M. & Gray, L.E.(1972). Dimensions and ultrastructure of toadfish gills. Biol. Bull. 143, 150-161.

Hughes, G.M. (1978). Morphology and morphometrics of fish gills. Atti Soc. Pelorit., Sc. Fis. Mat. e Nat., XXIV. pp333-335.

Hughes, G.M. (1984). General anatomy of the gills. In "Fish Physiology " (Hoar, W.S.& Randall, D.J.,eds), Academic Press, New York. Vol XA,pp1-71.

Ingram, G.A. (1980). Substances involved in the natural resistance of fish to infection - A review. J. Fish Biol. 16, 23-60.

Jackson, W.T. (1983). The Influence of Fish Population Densities on the Growth of Cyprinus Carpio (Linn.) and Larvae of Xenopus Laevis (Daudin) with particular reference to chemical factors: PhD. thesis Coventry (Lancaster) Polytechnic, England.

Jacobs, M.H. (1940). Some aspects of cell permeability to weak electrolytes. Cold Spring Harbour Symp. Quant. Biol. 8, 30-39.

Jhingran, V.G.(1985). Fish and fisheries of India 2nd edition, Hindustan publishing Corporation, Delhi, pp660.

Jhingran V.G. & Pullin, R.S.V. (1986). A hatchery manual for the common carp, Chinese and Indian major carps. Asian Development Bank, ICLARM, pp191.

Jordan, H.E. & Spidel, C.C. (1924). Studies on lymphocytes. II. The origin function and fate of the lymphocytes in fishes. J. Morph. 38, 529-549.

Kabata , Z. (1985). Parasites and Diseases of Fish Cultured in the Tropics. Taylor and Francis, London and Philadelphia. pp303.

Karlsson, L. (1983). Gill morphology in the zebrafish, Brachydanio rerio (Hamilton-Buchanan).J. Fish Biol. 23,511-524.

Karlsson-Norrgren, L., Runn, P., Haux, C. & Förlin, L. (1985a). Cadmium-induced changes in gill morphology of zebrafish,

Brachydanio rerio (Hamilton-Buchanan), and rainbow trout, Salmo gairdneri Richardson. J. Fish Biol. 27, 81-95.

Karlsson-Norrgren, L., Dickson, W., Ljungberg, O. & Runn, P. (1986b). Acid water and aluminium exposure: gill lesions and aluminium accumulation in farmed brown trout, Salmo trutta L. J. Fish Dis. 9, 1-9.

Karlsson-Norrgren, L., Björklund, I., Ljungberg, O. & Runn, P. (1986). Acid water and aluminium exposure: experimentally induced gill lesions in brown trout, Salmo trutta L. J. Fish Dis. 9, 11-25.

Kaushik, S.J. (1980). Influence of nutritional status on the daily patters of nitrogen excretion in the carp (Cyprinus carpio L.) and rainbow trout (Salmo gairdneri R.) Reprod. Nutr. Develop. 20, 1751-1765.

Klontz G.W., Stewart, B.C. & Eib, D.W. (1985). On the etiology and pathophysiology of environmental gill disease in juvenile salmonids. In: Ellis, A.E. edited, Fish and Shell Fish Pathology. Academic Press, London. pp199-210.

Kitzan, M.S. & Sweeny, P.R. (1968). A light and electron microscope study of the structure of protopterus annecteus epidermis. I. Mucus production Can. J. Zool. 46, 767-772.

Korgh,A, (1939). Osmotic regulation in aquatic animals. Cambridge Univ. Press,London and New York.

Kormanik,G.A. & Cameron,J.N.(1981).Ammonia excreation in the F.W.cat fish: the role of diffusion. Am. Soc. Zool. 21, 1042.

Kovács-Gayer, E. (1984). Histopathological differential diagnosis of gill changes with special regard to gill necrosis. Symp. Biol. Hung. 23, 219-229.

Kuhn, O & Koecke, H.U. (1956). Histologische und cytologische veranderungen der fischkieme nach einwirkung im wasser enthaltener schadigender substanzen. Z.Zellforsch. 43,611-643.

Kumar, S. & Pant, S.C. (1981). Histopathologic effects of acutely toxic levels of copper and zinc on gills, liver and kidney of Puntius conchonius (Ham.). Indian. J. Exp. Biol. 19, 191-194.

Lang, T., Peters, G., Holfmann, R. & Meyer, E. (1987). Experimental investigations on the toxicity of ammonia: effects on ventilation frequency, growth, epidermal mucous cells, and gill structure of rainbow trout Salmo gairdneri. Dis. Aquat. Org. 3, 159-165.

Larmoyeux, J.D. & Piper, R.G. (1973). Effects of water reuse on rainbow trout in hatcheries. Prog. Fish-cult. 35, 2-8.

Laurent, P. & Dunel, S. (1980). Morphology of gill epithelia in fish. Am. J. Physiol. 238, R147-R159.

Laurent, P. (1984). Gill internal morphology. In "Fish physiology " (Hoar, W.S. & Randall, D.J., eds). Academic Press, New York. Vol XA. pp73-183.

Leino, R.L. (1974). Ultrastructure of immature, developing and secretory rodlet cells in fish. Cell. Tiss. Res. 155, 367-381.

Leino, R.L. & McKormick, J.H. (1984). Morphological and morphometrical changes in chloride cells of the gills of Pimephales promelas after chronic exposure to acid water. Cell Tiss. Res. 236, 121-128.

Leino, R.L., Wilkinson, P. & Anderson, J.G. (1987). Histopathological changes in the gills of pearl dace Semotilus margarita and fathead minnows, Pimephales promelas, from experimentally acidified Canadian lakes. Can. J. Fish. Aquat. Sci. 44, 126-134.

Lloyd, R. and Herbert. D.W.M. (1960) The influence of carbon dioxide on the toxicity of un-ionized ammonia to rainbow trout (Salmo gairdnerii Richardson). Ann. appl. Biol. 45, 521-527.

Lloyd, R. & Orr, L.D. (1969). The diuretic responses by rainbow trout to sublethal concentrations of ammonia.Water Res. 3. 335-344.

Lloyd, R. & Swift, D.J. (1976). Some physiological responses by fresh water fish to low dissolved oxygen, high carbon dioxide, ammonia and phenol with particular reference to water balance. In Effects of Pollutants on Aquatic Organisms (A.P.M.Lockwood,

ed). Cambridge: Cambridge University Press. pp 47-71.

Maetz, J. & Garcia-romeu, F. (1964). The mechanism of sodium and chloride uptake by the gills of a fresh water fish, Carassius auratus II. Evidence for NH_4^+/Na^+ and HCo_3^-/Cl^- exchanges. J. Gen. Physiol. 47, 1209-1227.

Maetz, J. (1972). Branchial sodium exchange and ammonia excretion in goldfish, Carassius auratus. Effects of ammonia loading and temperature changes. J. Exp. Biol. 56, 601-620.

Maetz,J.(1973). Na2+/NH4+ , Na+/H+ exchanges on NH3 movement acrossthe gills of Carassius auratus.J. Exp. Biol..58.255-275.

Mackereth, F.J.H., Heron, J. & Talling, J.F. (1978). Water Analysis: Some revised methods for limnologists. Scientific publication NO.36. Ambleside, Cumbria, UK. Fresh water Biological Association. pp120.

Makarewich,W.,& Zydowo,M.(1962).comparative studies on some ammonia producing enzymes in the excretory organs of vertebrates. Comp.Biochem.Phisiol. 6, 269-275

Mallatt, J. (1985). Fish gill structural changes induced by toxicants other than irritants: A statistical review. Can. J. Fish. Aquat. Sci. 42, 630-648.

Mattey, D.L., Morgan, M. & Wright, D.E. (1979). Distribution and development of rodlet cells in the gills and pseudobranch of the

bass, Dicentrachus labrax (L). J. Fish Biol. 15, 363-370.

Marchetti, R.(1960).Nouvells études sur la toxicologie des poissons au point de vue du controls des eaux usées. Ann. Stat. Centr. Hydrobiol. Appl. 8, 107

Martinez,M.C.C. (1987). Studies on water soluble vitamin requirements in Cichlosoma uropthalmus (Gunther 1862). PhD thesis, pp. 211. University of Stirling. Stirling, Scotland.

Matthiessen, P. & Brafield, A.E. (1973). The effects of dissolved zinc on the gills of the stickleback, Gasterosteus aculeatus (L.) J. Fish Biol. 5, 607-613.

McDonald,D.G. & Wood, C.M. (1981). Branchial and renal acid and ion fluxes in the rainbow trout, Salmo gairdneri, at low environmental pH. J. Exp. Biol. 93, 101-118.

McDonald, D.G. (1983). The effects of H^+ upon the gills of fresh water fish. Can. J. Zool. 61, 691-703.

McFarland, W.N. & Norris, K.S. (1958). The control of pH by buffers in fish transport. California Fish and Game. 44, 291-310.

McLay,A.(1985).The pathology and experimental infections of *Ichthyophonus hoferi* and *Ichthyophthirius multifiliis* in rainbow trout *Salmo gairdneri*.PhD..Thesis.University of Aberdeen.pp.238.

Meade, J.W. (1985). Allowable ammonia for fish culture. Prog. Fish-cult. 47, 135-145.

Ministry of Technology (1968). Water pollution research 1967. Her Majesty's stationary office, London, England.

Ministry of Housing and Local Government. (1969). Fish Toxicity Tests. Her Majesty's Stationary Office, London. pp1-15.

Mitchell, S.J. & Cech, J.J. (1983). Ammonia caused gill damage in channel catfish, (Ictalurus punctatus):confounding effects of residual chlorine. Can. J. Fish. Aquat. Sci. 38, 16-22.

Mittal, A.K. & Munshi, J.S.D. (1974). On the regeneration and repair of superficial wounds in the skin of Rita rita (Ham.) (Bagridae, Pisces). Acta Anat. 88, 424-442.

Mittal, A.K. & Agarwal, S.K. (1977). Histochemistry of the unicellular glands in relation to their physiological significance in the epidermis of Monopterus cuchia (Synbranchiformes, Pisces). J. Zool. 182, 429-439.

Mommsen, T.P. (1984). Metabolism of the fish gill. In Fish Physiology (Hoar, W.S. & Randall, D.J. eds). Academic press, New York. Vol.XA pp

Murphy, T.P. & Brownlee, B.G. (1981a). Blue-green algal ammonia uptake in hypertrophic prairie lakes. Can. J. Fish. Aquat. Sci. 38, 1040-1044.

Murphy, T.P. & Brownlee (1981b). Ammonia volatilization in a hypertrophic prairie lake. Can. J. Fish. Aquat. Sci. 38, 1035-1039.

Murthy, A.S. (1986a). Toxicity of pesticides to fish. Volm.1, pp 117-145. CRC press, Inc.

Murthy, A.S. (1986b). Toxicity of pesticides to fish. Volm.2,pp 15-23. CRC press, Inc.

Nemòsok, J., Györe, K., Oláh, J. & Boross, L. (1984). Effects of NH_3 on blood glucose and catecholamine level, GOT, GPT, LDH-enzyme activity and respiration of fishes. Symp.Biol. Hung. 23, 209-217.

Nemoto,C.M. (1957) Experiments with Methods of air transport of live fish. Prog.Fish-cult.19,147-157.

Ogata, H. & Arai, S. (1985). Comparison of free amino acid contents, in plasma, whole blood and erythrocytes of carp, coho salmon, rainbow trout, and channel catfish. Bull. Jap. Soc. Scient. Fish. 51, 1181-1186.

Oronsaye, J.A.O. & Brafield, A.E. (1984). The effects of dissolved cadmium on the chloride cells of the gills of the stickleback, Gasterosteus aculeatus L. J. Fish Biol. 25, 253-258.

Payan, P. & Matty, A.J. (1975). The characterstics of ammonia excretion by a perfused isolated head of trout (Salmo gairdneri): effects of temperature and Co_2-free ringer. J. Comp. Physiol.96. 167-184.

Payan, P. (1978). A study of the Na^+ / NH^{4+} exchange across the gill of the perfused head of the trout (Salmogairdneri). J.Comp.Physiol. 124, 181-188.

Pequin,L.(1962).Les teneurs en azote ammoniacal du sang chez la Carpe (Cyprinus carpio L.,). Compt.Rrend. 255 1795-1797.

Pequin ,L.,& Serfaty,A. (1963). L'excretion ammoniacal chez un Téléostéen dulcicole: Cyprinus carpio L. Comp.Biochem.Physiol. 10, 315-324.

Post, G. (1983). Text Book of Fish Health. The Publications Inc. Ltd. New Jersey. pp 52-53 and 232.

Percy, L. R. (1970). Wandering cells in the epidermis of the goldfish. Proc. Univ. Newcastle Philp Soc. 1, 189-193.

Pickering, A.D. (1974). The distribution of mucus cells in the epidermis of the brown trout Salmo trutta (L.) and the char. Salvelinus alpinus (L.) J. Fish Biol. 6, 111-118.

Pickering, A.D. & Fletcher, J.M. (1987). Sacciform cells in the epidermis of the brown trout, Salmo trutta, and the arctic char, Salvelinus alpinus. Cell. Tiss. Res. 247, 259-265.

Pickering, A.D. & Macey, D.J. (1977). Structure, histochemistry and the effect of handling on the mucous cells of the epidermis of the char Salveliuns alpinus (L.) J. Fish Biol. 10, 505-512.

Pitts, R.F. (1964). Renal production and excretion of ammonia. Am.J.Med. 36, 720-742.

Pottinger, T.G., Pickering, A.D. & Blackstock, N. (1984). Ectoparasite induced changes in epidermal mucification of brown trout, Salmo trutta L. J. Fish Biol. 25, 123-128.

Rankin,J.C.& Davenport,J.A.(1981) Animalosmorogulation. PP. 202. Blackie, Glasgow and London.

Randall.D.J., Perry, S.F.& Heming T.A.(1982).Gas transfer and acid /Base regulation in salmonids. Comp. Biochem.Physiol.73B,93-103.

Randall, D.J. & Wright, P.A. (1987). Ammonia distribution and excretion in fish. Fish Physiol. Biochem. 3, 107-120.

Rao, T.S., Rao, M.S. & Prasad, S.B.S.K. (1975). Median tolerance limits of some chemicals to the fresh water fish "Cyprinus carpio". Indian J. Environ. Health. 17, 140-146.

Redner, B.D. & Stickney, R.R. (1979). Acclimation to ammonia by Tilapia aurea. Trans. Am. Fish. Soc. 108, 383-388.

Redner,B.D., Tomasso,R.J. & Simco, B.A.(1980). Short term allevation of ammonia toxicity by environmental sodium chloride in channel catfish (Ictalurus punctatus). J.Tenn.Acad.Sci.55,pp54.

Reimschuessel, R., Bennett, R.O., May, E.B. & Lipsky, M.M. (1987). Eosinophilic granular cell response to a microsporidian infection in a sergeant major fish, Abudefduf saxatilis (L.) J. Fish Dis. 10, 319-322.

Rice, S.D. & Stokes, R.M. (1975). Acute toxicity of ammonia to several developmental stages of rainbow trout, Salmo gairdneri. Fish. Bull. 73, 207-211.

Reichenbach-Klinke, H.H. (1967). Untersuchungen uber die einwirkung des ammoniakgehalts auf den fischorganismus. Arch. Fischereiwiss. 17, 122-132.

Roberts, R.J., Young, H. & Milne, J.A. (1971). Studies on the skin of plaice (Pleuronectes platess. L.).J.Fish Biol. 4,87-98.

Roberts, R.J. (1972). Ulcerative dermal necrosis (UDN) of salmon (Salmo salar L.). Symp. Zool. Soc. Lond. 30, 53-81.

Roberts, R.J., McQueen, A., Shearer, W.M & Young, H. (1974). The histopathology of salmon tagging. II. The chronic tagging lesions in returning adult fish. J. Fish Biol. 5, 615-619.

Roberts, R.J. (1978). Fish Pathology. Bailliere Tindall, London.

Robinette, H.R. (1976). Effects of selected sublethal levels of ammonia on the growth of channel cat fish Ictalurus punctatus Porg. Fish-cult. 38, 26-9.

Roubal, F.R., Bullock, A.M., Robertson, D.A. & Roberts, R.J. (1987). Ultrastructural aspects of infestation by Ichthyobodo necator (Henneguy, 1883) on the skin and gills of the salmonids Salmo salar L. and Salmo gairdneri Richardson. J. Fish Dis. 10, 181-192.

Rubin, A.J. & Elmaraghy, G.A. (1977). Studies on the toxicity of ammonia, nitrite and their mixtures to guppy fry. Water Res. 11, 927-935.

Saha, K.C., Sen, D.P. & Mazumdar, P. (1956). Studies on the mortality of spawn and fry of Indian major carps during transport. III. Effect of inimical substances from decomposition of metabolic products in the medium on spawn life and their control. Indian J. Fish. 3, 135-140.

Scheiner,D.(1976).Determination of ammonia and Kzeldahl nitrogen by indophenol method . Water res.10,31-36.

Schulze-Wiehenbrauck, H. (1976). Effects of sublethal ammonia concentrations on metabolism in juvenile rainbow trout (Salmo gairdneri Richardson) Ber. D Tsch. Wiss. Komm. Meeresforsch. 24, 234-250.

Sheehan, R.J. & Lewis, W.M. (1986). Influence of pH and ammonia salts on ammonia toxicity and water balance in young channel catfish. Trans. Am. Fish. Soc. 115, 891-899.

Sibbing, F.A. & Uribe, R. (1985). Regional specializations in the oro-pharyngeal wall and food processing in the carp (Cyprinus carpio L.) Neth. J. Zool. 35, 377-422.

Smart, G.R. (1975). The Acute Toxic mechanism of Ammonia to Rainbow trout (Salmo gairdneri). PhD. thesis. University of Bristol. England.

Smart, G.R. (1976). The effects of ammonia exposure on gill structure of the rainbow trout (Salmo gairdneri). J. Fish. Biol. 8, 471-475.

Smart, G.R. (1978). Investigations of the toxic mechanisms of ammonia fish-gas exchange in rainbow trout (Salmo gairdneri). J. Fish Biol. 8, 471-475.

Smart, G.R. (1981). Aspects of water quality producing stress in intensive fish culture: In Fish and Stress edited by Pickering, A.D., Academic Press, London. pp277-289.

Smith, H.W. (1929). The excretion of ammonia and urea by the gills of fish. J. Biol. Chem. 81, 727-742.

Smith, C.E. & Piper R.G. (1975). Lesions associated with chronic exposure to ammonia. In Rebelin W.E. & Migaki G. (eds). The

Pathology of Fishes. University of Wisconsin Press, Madison, WI. pp497-514.

Smith, H.E. (1975). Eosinophil Granule Cells of Salmonids. MSc dissertation, Institute of Aquaculture, University of Stirling, Stirling, UK. pp 45.

Smith, C.E. (1984). Hyperplastic lesions of the primitive meninx of fat head minnows, Pimephales promelas, induced by ammonia; special potential for carcinogen testing. Nat. Cancer Inst. Monogr. 65, 119-125 (USA).

Soderberg, R.W., McGee, M.V., Grizzle, J.M. & Boyd, C.E. (1984). Comparative histology of rainbow trout and channel catfish growth in intensive static water aquaculture. Prog. Fish-cult. 46, 195-199.

Soderberg, R.W. (1985). Histopathology of rainbow trout Salmo gairdneri Richardson, exposed to diurnally fluctuating un-ionized ammonia levels in static-water ponds. J. Fish Dis. 8, 57-64.

Sousa, R.J., Meade, T.C. & Wolke, R.E. (1974). Reduction of ammonia toxicity by salinity and pH manipulation. Proceedings of the Fifth Annual Workshop, World Mariculture Society 5, 343-355.

Sousa. R.J. and Meade, T.L. (1977). The influence of ammonia on the oxygen delivery system of coho salmon hemoglobin. Comp .

Biochem. Physiol. 58 A, 23-28.

Spotte, S.H. (1970). Fish and Invertebrate Culture: Water management in closed Systems. Wiley-interscience, John Wiley and Sons, Inc. London. pp131.

Sprague, J.B. (1973). The ABC's of pollutant bioassay using fish. American Soc. for Testing and Materials (ASTM) STP. 528, 6-30.

Srinivasan, R., Chacko, P.I. & Valsan, A.P. (1955). A preliminary note on the utility of sodium phosphate in the transport of fingerlings of Indian carps. Indian J. Fish. 2, 77-83.

Srewart,B.C.(1983).Environmental gill disease in Salmonids: The role of environmental unionised ammonia. MSc.,Thesis.Graduate school,University of Idaho.pp.71.

Stirling, G.A. & Kakkar, V.V. (1969). Cells in the circulating blood capable of producing connective tissue. Br. J. Exp. Path. 50, 51-55.

Stirling, H.P. (Editor). (1985). Chemical and Biological Methods of Water Analysis for aquaculturists. Institute of Aquaculture, University of Stirling, Stirling, Great Britain. pp118.

Stumm, W. & Morgan, J.J. (1981). Aquatic chemistry. 2nd edition. John Wiley and Sons, New York.

Subramanian, S. (1983). Eradication of fish by application of ammonia. Aquaculture. 35, 273-275.

Szumski.,D.S., Barton,D.A.,Putnum,H.D. & Polta, R.C. (1982). Evaluation of EPA un-ionoized ammonia toxicity criteria. Journal Water pollut.Control fed. 54,281-291.

Thurston, R.V., Russo, R.C. & Smith, C.E. (1978). Acute toxicity of ammonia and nitrite to cut-throat trout fry. Trans. Am. Fish. Soc. 107, 361-368.

Thurston, R.V., Russo, R.C. & Emerson, K. (1979). Aqueous ammonia equilibrium-tabulation of percent un-unionized ammonia. United States Environmental Protection Agency, Ecological Research Series EPA. 600/3, 79-91.

Thurston, R.V., Russo, R.C. & Vinogradov, G.A. (1981). Ammonia toxicity to fishes. Effect of pH on the toxicity of the unionized ammonia species. Environ. Sci.&Technol. 15, 837-840.

Thurston, R.V. & Russo, R.C. (1983). Acute toxicity of ammonia to trout. Trans. Amer. Fish. Soc. 112, 696-704.

Thurston,R.V.,Russo,R.C. & Phillips,G.R.(1983). Acute toxicity of ammonia to fathead minnows. Trans.Am.Fish.Soc. 108,383-388.

Thurston, R.V., Russo, R.C., Luedtke. R.J., Smith, C.E., Meyn, E.L., Chakoumakos, C., Wang, K.C. & Brown, C.J.D. (1984).

Chronic toxicity of ammonia to rainbow trout. Trans. Am. Fish. Soc. 113, 56-73.

Thurston,R.V., Russo,R.C.,Meyn,E.L.& Zajdel,R.K.(1986).Chronic toxicity of ammonia to fathead minnows.Trans.Am.Fish. Soc.115, 196-207.

Tomasso, J.R., Goudie, C.A., Simco, B.A. & Davis, K.B. (1980). Effects of environmental pH and calcium on ammonia toxicity in channel catfish. Trans. Am. Fish. Soc. 109, 229-234.

Tóth, E.O., Gulyás, P. & Oláh, J. (1982). Effects of temperature on growth, food conversion, and survival of sheathfish (Silurus glanis L.) and common carp (Cyprinus carpio) at sublethal ammonia concentration. Aquacultura Hungarica (Szarvas) III, 51-56.

Trussell, R.P. (1972). The percent unionized ammonia in aqueous ammonia solutions at different pH levels and temperatures. J. Fish. Res. Board Can. 29, 1505-1507.

Tucker, L., Boyd, C.E. & McCoy, E.W. (1979). Effects of feeding rate on water quality, production of channel catfish, and economic returns. Trans. Am. Fish. Soc. 108, 389-396.

Tucker, C.S. (Editor). (1985). Channel Catfish Culture. Elsevier, Amsterdam. pp1-647.

USEPA (U.S. Environmental Protection Agency) (1985). Ambient water quality criteria for ammonia. U.S. EPA 440/5-85-001. Office of water regulations and standards, Washington D.C.

Vallejo, A.N. (1987). An Ultrastructural Study of the Response of Eosinophil Granule Cells to Aeromonas salmonicida Extracellular Products (ECP) on Histamine Liberators with Notes on the Histopathology of ECP Baths in Rainbow trout Salmo gairdneri. MSc dissertation: Institute of Aquaculture, University of Stirling, Stirling, UK. pp 95.

Vamos, R. (1963). Ammonia poisoning in carp. Acta biol. Szeged. 9, 291-297.

Vamos, R. & Tasnadi, R. (1967). Ammonia poisoning in carp. 3. The oxygen content as a factor influencing the toxic limit of ammonia. Acta Biol. Szeged. 13, 99-105.

Vaas, K.F. (1952). Preliminary report on air transport of live fish in sealed tins under oxygen pressure. Proc. Indo-pacific Fish. Council, Sec. 11, 119-128.

Van Oosten, J. (1957). The skin and scale. In The Physiology of Fishes. Vol.1 (Brown, M.E., ed). New York: Academic Press. pp207-244.

Vellas-Clos, F. (1973). Research on ureogenesis in fresh water teleosts. Thesis Docteur es-sciences naturelles, University Paul sabatier de Toulouse. 155,158,160,162.217.

Vellas- Clos, F. and Serfaty, A. (1974). 'ammoniaque et l'ure'e Chez un Te'le'oste'en d'ean douce: la carpe (Cyprinus carpio L.). J. Physiol. Paris.68. 591-614.

Visek, W. J. (1968). Some aspects of ammonia toxicity in animal cells. J. Dairy Science. 51, 286-295.

Watts,R.L.& Watts,D.C. 1974. Nitrogen metabolism in fishes: In Chemical Zoology.Vol. 8.pp.369-446. Edited by M.Florkin and B.T.Scheer.Academic press Inc.,New York.

Walker,C.O. & Schenker,S.(1970).Pathogenesis of hepatic encephalopathy - with
special reference to the role of ammonia. Am.J.Clin.Nutr. 23, 619-632.

Weiler, R.R. (1979). Rate of l ss of ammonia from water to the atmosphere, J. Fish. Res. Boar Can. 36, 685-689.

Whitear, M. (1986). The skin of fishes including cyclostomes; Epidermis: In Biology of the Integument Vol.2 Vertebrates (Bereiter-hahn,J., Matoltsy, A.G. & Richards, K.S. Eds.,) Springer-verlag Berlin Heidelberg. pp8-38.

Whitear, M. & Mittal, A.K. (1986). Structure of the skin of Agonus cataphractus (Teleostei) J. Zool. Lond. A210, 551-574.

Wuhrmann,K.,Zehender,F.and Woker,H. (1947).Über die fischereibiologische bedeutung des ammonium-und Ammoniakgehaltes fliessender Gewässer. Z. natur. Ges. Zurich. 92,198-204.

Wuhrmann,K.& Woker,H.(1948).Experimentelle untersuchungen über die ammoniak -und blausäurevergiftung. Schweiz Z.Hydrol. 11, 210-244.

Wood, J.D. (1958). Nitrogen excretion in some marine teleosts. Can. J. Biochem. Physiol. 36, 1237-1242.

Zaccone, G. & Cascio, P.L. (1979). Histological and histochemical study of the epidermis of Ophisurus serpens (Anguilliformes, Pisces) under normal and stressed conditions. Riv. Biol. Norm. Patol. 5, 201-222.

Zaccone, G. (1980a). Structure and histochemistry of the epidermis in the red piranha Serrasalmus nattereri (Kner, 1860) (Characidae, Teleostei). Cell Mol. Biol. 26, 75-84.

Zaccone, G. (1980b). On the occurrence of eosinophilic granular cells in teleost epidermis. A review. Zool. Jb. Anat. 104, 139-143.

APPENDICES

APPENDIX-1

The anatomical structure and the arrangement of the gill filaments and lamellae does not differ much between carp and trout, but a few marked differences do occur in the morphological features of carp and trout gills. Some of these differences, as noted in the present investigations, are described in this appendix. The most conspicuous difference between the carp and trout gills appears to be the epithelial microridge pattern on the gill surface. In carp well defined concentric whorls of microridges decorate the epithelial cells of the filament and arch epithelium but the lamellar epithelium is devoid of such microridges in general.

Secondly the carp gill filaments possess numerous regularly spaced tastebud cells on the efferent side of the gill filaments extending from the proximal end to about half the length of the filament. On the other hand the trout gills do not posses any taste bud cells on the filaments and the microridges do not form regular patterns as seen in the case of carp, but are uniformly present on both the lamellar and filament epithelium. The gill epithelium in general is richly endowed with normal mucous cells which are commonly involved in mucus secretions. The scanning electron micrographs from A to F presented here represent some of the structural features and differences discussed above.(Plates: A to D carp gills, E and F - trout gills).

Plate A

(1) A Scanning Electron Micrograph (SEM) of the normal gill structure of common carp displaying filaments (primary lamellae) with regularly and equidistantly arranged lamellae (secondary lamellae), 200 X.

(2) SEM of the normal carp gill lamellae (700 X)

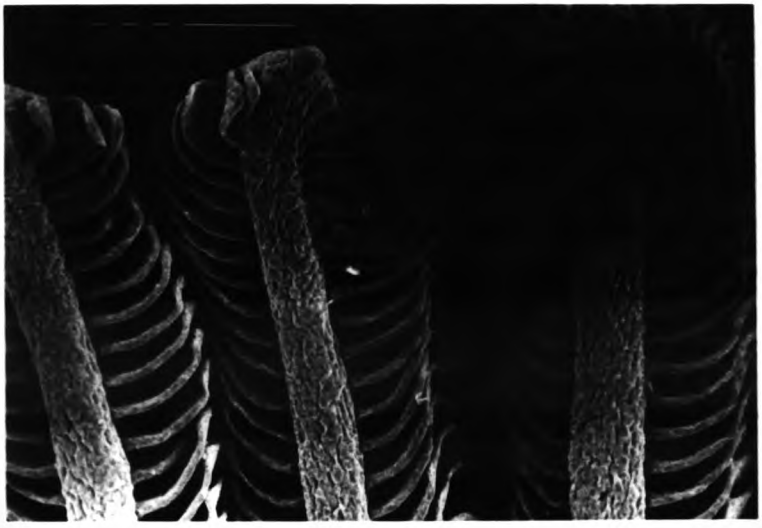


Plate B

(1) SEM of the normal carp gill filaments with regularly spaced taste bud cells (arrowed) and lamellae (100 X)

(2) SEM of a taste bud cell of the gill filament with its characteristic apical opening and receptor processes (9000 X)

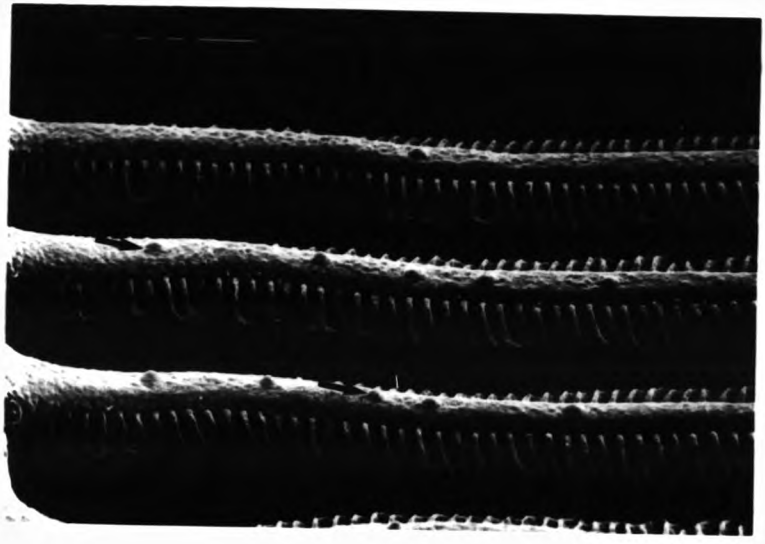


Plate C. SEM of epithelial cell microridge patterns on the gill epithelium

(1) Note the characteristic filament epithelial microridges of the cells (6000 X)

(2) Lamellar epithelial of carp showing the absence of typical filamental microridge pattern (6000 X)

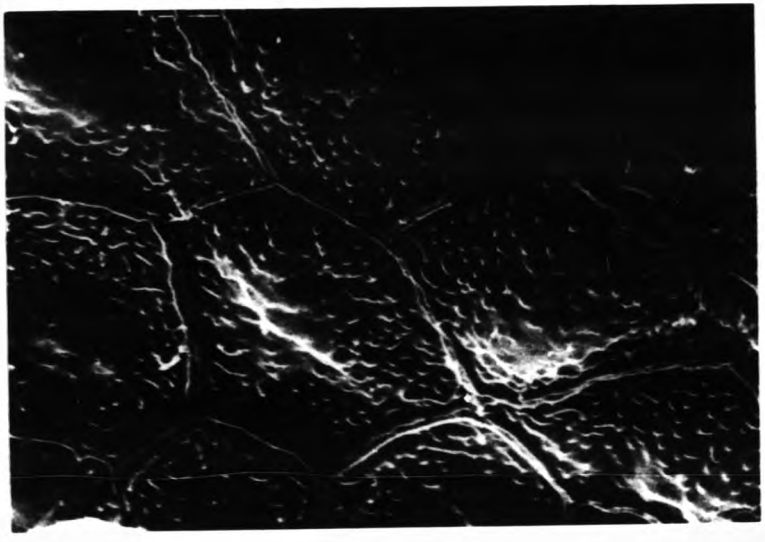


Plate D

(1) Normal epidermal mucous cell pores and mucous secretions (1600 X)

(2) Normal gill arch epithelial mucous pores and mucous secretions, (1600 X).

(Note the active mucous secretion in both the cases and the normal mucous cell opening)

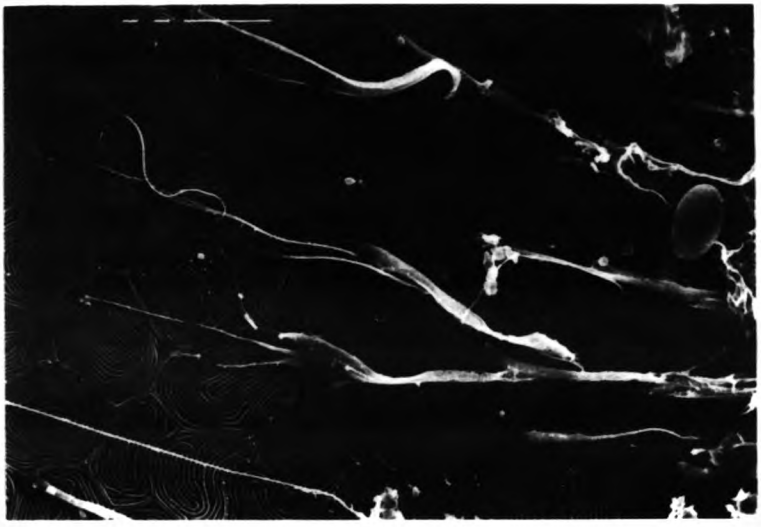


Plate E.

(1) SEM of normal trout gill filament with normal and regularly spaced lamellae, 900X.

(2) SEM of normal trout gill lamellae showing uniform microridge pattern both on the lamellar (L) and filament epithelium (F), 3500X.

Plate F

(1) A SEM of filament epithelium of trout displaying the process of normal mucous production,(3000X)

(2) A SEM of the filament epithelium of trout showing a thin mucous layer on the filament.

APPENDIX 2

Processing routine for the automatic tissue processor

50% methylated spirits	1 hour
80% methylated spirits	2 hours
100% methylated spirits	2 hours
100% methylated spirits	2 hours
100% methylated spirits	2 hours
Absolute alcohol	2 hours
Chloroform	2 hours
Chloroform	1 hour
Chloroform	1 hour
Paraffin wax	2 hours
Paraffin wax	2 hours

APPENDIX 3

Haematoxylin-Eosin staining

(1) Bring section to water by baths in:

Xylene	5 minutes
Absolute alcohol	2 minutes
Methylated spirit	1.5 minutes

(2) Haematoxylin - 10 minutes

(3) Wash in tap water -1 minute

(4) Differentiate in Acid Alcohol 1%

(5) Wash in tap water- 1 minute

(6) Scott's tap water substitute - 1 minute

(7) Eosin - 3 minutes

(8) Methylated spirit - 30 seconds

(9) Absolute alcohol - 2 minutes

(10) Absolute alcohol - 1.5 minutes

(11) Xylene - 5 minutes

(12) Mount in synthetic resin

APPENDIX 4

Alcian blue staining pH 2.5

Alcian blue stain : Alcian blue 0.5 g
Glacial acetic acid 3 ml
Distilled water to 100 ml

(1) Bring sections to water
(2) Alcian blue stain - 20 minutes
(3) Rinse in distilled water
(4) Wash in running water - 5 minutes
(5) Counter stain with 1% aqueous neutral red
(6) Methylated spirit - 30 seconds
(7) Absolute alcohol - 2 minutes
(8) Absolute alcohol - 1.5 minutes
(9) Xylene - 5 minutes
(10) Mount in synthetic resin

Reading: Acid mucopolysaccharides : green
Cell nuclei : red
Background : yellow

APPENDIX 5

Periodic Acid - Schiff's (P.A.S.) Reaction

(1) Sections to water

(2) 1% Periodic acid - 10 minutes

(3) Wash in tap water - 5 minutes

(4) Schiff's reagent - 20 minutes

(5) Wash in tap water

(6) Haematoxylin - 5 minutes

(7) Wash in tap water

(8) Differentiate in 1% Acid Alcohol

(9) Blue in Scott's tap water substitute

(10) Wash in tap water

(11) Methylated spirit - 30 seconds

(12) Absolute alcohol - 1 minute

(13) 0.3% tartrazine in cellosolve - 3 minutes

(14) Absolute alcohol - 1.5 minutes

(15) Xylene - 5 minutes

(16) Mount in synthetic resin

Results: P.A.S. positive : Red or magenta

Nuclei : Blue

APPENDIX-6

Staining techniques for Rodlet cells in wax sections (modified from Dougherty 1981):

Methylene blue- Basic fuchsin staining

Stock solutions:

Methylene blue- 0.13 g dye in 100 ml distilled water
Basic fuchsin- 0.13 g dye in 100 ml distilled water
0.2M Sorensen phosphate buffer (pH 7.2-7.4)- mixture of 6.5 ml sodium phosphate monobasic and 43.5 ml sodium phosphate dibasic made to 100 ml volume in distilled water.

Stain working solution: Mixture of 12 ml methylene blue, 12 ml basic fuchsin, 21 ml buffer and 15 ml 95% ethanol. Filter and stored in dark bottle, stain good for 5 days.

procedure:

(1) bring sections to water
(2) stain with working solution for 3 minutes
(3) wash excess stain in running water
(4) one quick dip in acid-alcohol
(5) wash thoroughly in running water (check under the microscope, restain if necessary for 1 min)
(6) dehydrate
(7) clear in xylene and mount