EXCRETION OF UREA BY FISH EXPOSED TO DIFFERENT CONCENTRATIONS OF AMBIENT AMMONIA

Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY
KENNETH R. OLSON

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THESIS

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ABSTRACT

EXCRETION OF UREA BY FISH EXPOSED TO DIFFERENT CONCENTRATIONS OF AMBIENT AMMONIA

By

Kenneth R. Olson

Urea has long been known to be an excretory product in fish; however, its mode of synthesis and the role of urea production in ammonia detoxification are not well understood. Increased ambient ammonia concentrations give rise to increased blood ammonia levels in teleosts, and under these conditions the possible role of the synthesis of urea or any other nitrogenous compound in detoxification of ammonia can be ascertained.

Experiments were conducted to determine if any species variability to ammonia toxicity exists between two freshwater teleosts that are able to inhabit distinctly different environments. Rainbow trout (Salmo gairdneri) that require relatively clear water and goldfish (Carassius auratus) which have a tolerance for stagnant water were used for the range of ecological requirements. Information on the pattern of waste nitrogen excretion by trout was obtained

by measuring total nitrogen, ammonia nitrogen, urea nitrogen and protein nitrogen excreted during a 24-hour period.

Trout subjected to increased ambient ammonia concentrations showed a decrease in total nitrogen excreted; concomitantly there was a decrease in nitrogen excreted as ammonia. Except for an initial increase in urea excretion at low levels of ambient ammonia, urea and protein nitrogen excretion rates remained unchanged as ambient ammonia increased. Ninety-four per cent of the total nitrogen excreted by trout was as ammonia, urea and protein, and trout acclimated to high ammonia levels for extended periods of time showed no increase in urea excretion above that of trout acclimated to very low ammonia for the same time period.

A considerable increase in rate of urea excretion was noted in goldfish when ambient ammonia concentration increased. The time course for the change in rate of urea excretion in response to a change in ambient ammonia levels is nearly instantaneous. Urea excretion rate by goldfish is dependent on the ambient ammonia concentration which prevails and is independent of acclimation concentrations and duration of acclimation.

An apparent species difference exists between goldfish and rainbow trout which is noted not only physiologically but behaviorally as well. The physiological response by trout to increased ammonia

levels was a very limited increase in urea excretion and occurred only at very low levels, whereas goldfish excreted increased amounts of urea as ambient ammonia increased. Under similar ammonia conditions goldfish excrete more urea per gram of fish than trout. The LD_{50} for trout is around 8μg ammonia/ml, whereas goldfish were little affected by 25μg ammonia/ml. Hyperexcitability observed in trout at 3μg ammonia/ml was absent in goldfish even at 25μg ammonia/ml. Histological studies indicated that in goldfish exposed to 25μg ammonia/ml for 8 weeks the severity of gill lamellae deterioration was much less than that observed in rainbow trout exposed to 5μg ammonia/ml for 8 weeks.

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By

Kenneth R. Olson

A THESIS

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Dedicated to my wife, Marilyn, for her patience, understanding and many long hours of help in the completion of this dissertation.

Dedicated also to my parents, Robert and Mildred Olson, whose interest in learning and encouragement made my education possible.

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INTRODUCTION AND LITERATURE REVIEW

Dietary nitrogen, which is necessary for normal growth, development and function of organisms, is catabolized through various metabolic pathways into a toxic end product, ammonia. Although ammonia is commonly found in metabolically active tissues, its concentration must be rigidly controlled to prevent toxic effects on the tissues.

The deleterious effects of ammonia on an organism can be alleviated in three ways: (1) excretion of ammonia at a rate equal to its formation, (2) conversion of ammonia to a less toxic compound, or (3) possible alteration of the sensitivity of an animal to elevated tissue ammonia levels. The capability of an organism to alter its ammonia sensitivity is unknown and, due to the diversity of parameters involved, attempts to quantitate sensitivity levels usually leads to qualitative and subjective observations. To the author's knowledge there are no data present in the literature relative to this point.

Excretion

Excretion of ammonia at a rate consistent with its formation (ammonotelism) is accomplished rather easily in aquatic animals. Smith (1929) and others more recently have demonstrated the role of branchial excretion of ammonia by fish. In fresh water rainbow trout, Fromm (1963) reported that more than 95% of the total waste nitrogen was excreted via the gills and that ammonia nitrogen comprised about 60% of the total nitrogen excreted by the fish. Ammonia excretion via the gills appears to be mainly by passive diffusion (Goldstein, Forster, and Fanelli, 1964; Fromm and Gillette, 1968) and would require little energy if an adequate blood to water diffusion gradient is maintained. This low energy requirement for excretion of ammonia, coupled with the fact that in many instances energy is derived from ammonia formation through deamination reactions (White, Handler and Smith, 1964), makes this mechanism of ammonia excretion highly beneficial to the animal.

Conversion

Ammonia in some organisms can be converted to less toxic compounds which can then be either excreted directly or stored until conditions are favorable for their removal. The two most important nitrogenous compounds which are synthesized by animals

are urea and uric acid. In teleoses the amount of nitrogen excreted in the form of uric acid is extremely small (Prosser and Brown, 1966) and is considered to be insignificant. Other nitrogen containing compounds such as amino acids, purines, trimethylamine oxide (TMAO), creatine and creatinine are also formed, but generally to a very small extent (Cragg, Balinsky and Baldwin, 1961; Prosser and Brown, 1966).

The main mechanisms of urea synthesis have been reviewed in detail by Hoar and Randall (1969), and they give a thorough discussion of the biochemical synthetic steps involved which were elucidated by White, Handler and Smith (1964). Three mechanisms are briefly discussed.

Dietary Arginine Catabolism

Arginine is converted, in the presence of arginase, to urea and ornithine, and the conversion requires no net expenditure of energy. Of the three mechanisms, dietary arginine degradation in fish probably accounts for the formation of the least amount of urea, due to the fact that arginine is an essential amino acid and cannot be synthesized by teleosts (Hoar and Randall, 1969).

Ornithine Cycle Activity

In the ornithine cycle two molecules of ammonia (NH_3) combine with one molecule of carbon dioxide to form urea and water with the net expenditure of 4 ATP's. Whether the full complement of ornithine cycle enzymes is present or not in teleosts is somewhat debatable. Hunter (1929) and Cvancara (1969) report that arginase activity in teleosts is commensurate with urea production by ornithine cycle activity, but Brown and Cohen (1960) were unable to detect any carbamoyl phosphate synthetase or ornithine transcarbamylast (two key enzymes for ornithine cycle activity); and they concluded that while arginase was present in sufficient amounts, the ornithine cycle was not functional in teleosts. More recent work (Huggins, Skutsch and Baldwin, 1969) has indicated that the ornithine cycle may be functional in some teleosts and evidence is given showing that some enzyme activity for all of the enzymes associated with operation of the cycle is present in most of the teleosts studied, including rainbow trout. Enzyme activities for teleost tissues ranged from one to three orders of magnitude lower than those found in amphibians as Rana angloensis, R. catesbeiana and R. temporaria.

Purine Metabolism

For purine synthesis, two molecules of ammonium (NH_4^+) are combined with glycine, aspartate and ribose-5-phosphate to

form inosinic acid and fumarate with an energy expenditure of 9 ATP's. Purine catabolism involves the breakdown of purines through xanthylic acid, uric acid and other intermediates to urea. Since purines are a necessary component of fish tissues, it follows that the synthetic machinery is present and operative for production of these compounds. The ultimate result of purine catabolism yields a net loss of four atoms of nitrogen in two molecules of urea. That some, if not all, of the enzymes necessary for catabolism of purines to urea (xanthine oxidase, uricase, allantoinase and allantoicase) are present in teleosts is indicated by the work of Cvancara (1969). He found relatively high levels of uricase activity in 19 species of fresh water teleosts and stated that preliminary results indicated levels of allantoinase and allantoicase were similar to that of uricase.

There is considerable evidence that the mode of excretion of waste nitrogen by animals is a labile characteristic. Brown, Brown and Cohen (1959) have observed a shift from ammonotelism to ureotelism by analyzing ornithine cycle enzyme activities in Rana catesbeiana tadpoles undergoing metamorphosis. Their data indicated that a de novo synthesis of ornithine cycle enzymes occurred during metamorphosis. McBean and Goldstein (1967) found a five-fold increase in urea excretion by Xenopus laevis adapted to

300 mOs/liter saline, and their data also indicated a de novo synthesis of ornithine cycle enzymes. When the mudskipper, Periopthalmus sobrinus, was removed from water for 12 hr, an increase in urea excretion was observed (Gordon et al., 1969). It is not known whether this increase was due to increased urea synthesis of to some factor such as increased active secretion of urea by the kidney, such as is known to occur in the South American frog, Leptodactilus occellatus (Carlisky, Botbol and Barrio, 1968), or to decreased active urea reabsorption as occurs in elasmobranchs (Smith, 1957).

RESEARCH RATIONALE

The purpose of this study was to partially block ammonia excretion in two species of ammonotelic teleosts and to determine the pattern of nitrogen excretion under these conditions. The experimental design was similar to that used by Fromm and Gillette (1968).

Fish were subjected to various concentrations both above and below the control levels of ambient ammonia for short periods of time or to elevated ambient ammonia for extended periods of time in an attempt to obtain data which would provide answers to the following questions:

1. Is there a species difference between Salmo gairdneri and Carassius auratus with regard to patterns of nitrogen excretion?
2. When the blood ammonia is elevated, how is the overall nitrogen excretion pattern altered in the two species?
3. Will any other nitrogenous compound be excreted in greater amounts than normal to compensate for a decrease in ammonia excretion when ambient ammonia is increased?

4. If decreased ammonia excretion is compensated for by excretion of another nitrogen containing waste, what is the time course for this compensation and is the time course consistent with possible induction of enzymes associated with either increased ornithine cycle activity or purine metabolism?

MATERIALS AND METHODS

Experimental Animals

Rainbow trout (Salmo gairdneri) weighing 50 to 100 grams were obtained from the Department of Natural Resources, Fish Research Laboratory at Grayling, Michigan. Except for one small group which were transported in plastic bags, the trout were transported to East Lansing, Michigan, in an insulated metal tank, the inside of which had been coated with nontoxic paint. En route the water was mechanically aerated and ice added to the water to insure an optimum temperature of around 13 C.

At Michigan State University the trout were stored in 300-liter fiberglass lined wooden tanks and maintained at 13 ± 1 C with a 14 hr daily photoperiod. The water was aerated and continually flushed with tap water from which iron and chlorine had been removed. Twice a week the fish were fed commercial trout pellets (3/16 inch).

Seven days prior to experimentation the fish were transferred to 100-liter tanks and starved to allow the nitrogen excretion

to approach a steady state (Fromm, 1963). Water, light, and temperature conditions remained as noted above.

Fifty 1- to 4-gram goldfish (Carassius auratus) used in this experiment were purchased at a local pet shop and maintained in two 20-gallon aquariums containing 36 liters of water at 20-23 C. The water was continually aerated. To insure control of the ammonia level, one-half of the water volume was removed on alternate days and replaced with aged tap water containing the desired concentrations of ammonia as ammonium chloride. The aquarium water was also cycled through an external filter containing glass wool to remove organic debris. All goldfish were fed finely ground trout pellets daily until one week before experimentation, at which time feeding was discontinued.

Experimental Design

Trout

Starved fish were individually placed in weighed, covered, 19.5 × 14.3 × 9.5 cm plastic containers containing 1 kg of aged, aerated tap water with the following ammonia concentrations: 0, 3, 5, or 8 μg ammonia per ml. These initial ammonia concentrations were chosen to give a range from a very low ammonia level to near the LD_{50} for trout. The containers were covered, then again weighed

to determine fish weight. Airstones were placed in each container to insure adequate oxygen, and an opaque curtain was drawn in front of the containers to prevent excitation of the fish due to other activity in the room. Temperature was maintained at 12 ± 1 C.

After 24 hr the fish were removed from the containers and 25 ml aliquotes of the ambient water were taken for analysis of total nitrogen, ammonia nitrogen, urea nitrogen, protein nitrogen and pH. Data from all fish that died during the 24 hr were discarded.

The average ambient ammonia concentration was determined from the initial ammonia concentration and the ammonia concentration found after 24 hr according to equation (1).

$$\text{Average Ambient Ammonia Concentration} = \frac{\left(\text{initial ammonia concentration}\right) + \left(\text{24 hr ammonia concentration}\right)}{2} \qquad (1)$$

When trout are placed in plastic containers containing 0 μg ammonia/ml for 24 hr, the ammonia level rises considerably due to the ammonia excreted by the fish. In an attempt to measure the effect of very low ammonia concentrations (i.e., < 0.5 μg ammonia/ml) on urea excretion, seven trout were placed in 1 kg of water in plastic containers to which enough Permutit (see Appendix IIA) had been added to cover the bottom of the container to a depth of approximately 2 mm. After 24 hr the trout were removed and the

ambient water was analyzed for urea nitrogen. To obtain comparative data, the seven trout used in the above experiment were fed for 7 days, starved for 7 days, and then returned to the plastic containers, which this time contained 1 kg of water to which no Permutit was added. Between the 20th and 24th hours of the experiment five of the seven fish died; however, ambient water from all seven fish was analyzed for urea and ammonia nitrogen. Average ambient ammonia concentrations were determined.

Two groups of 10 trout each were placed in tanks containing 92 liters water at 13 C. For one group, tap water was continually circulated through the tank at a rate of approximately 600 ml/min, and the ammonia levels were kept at or below 0.5 μg ammonia/ml. The other group of fish was exposed to elevated ammonia levels by changing two-thirds of the water, on alternate days, with aged tap water to which ammonium chloride had been added to achieve the desired ammonia level (5 μg ammonia/ml). After 4 weeks exposure the trout were individually placed in plastic containers containing 1 kg water and exposed to an initial ammonium concentration of 0 μg ammonia/ml for 24 hr. At the end of the experimental period the fish were returned to their respective tanks and water samples from the plastic tanks were analyzed for urea and ammonia nitrogen. Exposure of the acclimated fish to 5 μg ammonia/ml was continued

for an additional 4 weeks, at which time the gills were prepared for histological examination.

Goldfish

Twenty goldfish were placed in 36 liters of aged tap water in one of two 20 gal aquariums. The average ammonia level was kept under 0.5 μg ammonia/ml by changing half of the aquarium water with aged aerated tap water. The remaining 30 fish were placed in the other aquarium, and the ammonia levels maintained at 3 μg ammonia/ml for 5 days and immediately followed with 5 μg ammonia/ml for an additional 21 days. The mortality of rainbow trout at this level of ambient ammonia is low, and it was assumed that mortality rate would also be low for goldfish.

After 26 days groups of two or three fish (depending on the size of the fish) were taken from each aquarium and placed in weighed 10.6 × 8.5 × 7.0 cm staining dishes containing 250 g water with 5 μg ammonia/ml. The dishes were again weighed to give by difference the total weight of the fish in each dish. After 24 hours the fish were returned to their respective aquariums and duplicate 25 ml aliquotes of water from the staining dishes were analyzed each for ammonia nitrogen, urea nitrogen and pH. The dishes were uncovered in lieu of mechanical aeration, and water loss due to

evaporation was minimal. The photoperiod during these experiments was variable.

The ambient ammonia concentration for fish previously exposed to 5 μg ammonia/ml was then raised to an initial 25 μg ammonia/ml and this concentration was maintained for 20 days. On the 20th day the experimental (high ammonia acclimated) and control (low ammonia acclimated) fish were placed in 250 g of aged tap water in the weighed staining dishes, and reweighed to obtain fish weight. In an attempt to keep the bath ammonia level low, no exogenous ammonia was added to the water, and the fish were returned to their respective aquariums after 12 hr. Twenty-five ml aliquotes of the water samples from the staining dishes were again analyzed for ammonia nitrogen, urea nitrogen, and pH. The 24 hr excretory nitrogen values were calculated by multiplying the amount of nitrogen excreted in 12 hr by two.

The experimental and control goldfish remained in their respective aquariums for 10 additional days under the same conditions as above, at which time they were again placed in the 250 g of ammonia-free water and the weight of the fish in each dish determined as before. Ammonia levels were maintained at a minimum by the addition of approximately 5.4 g of Permutit (a cation exchange resin selective to ammonia) previously rinsed with water. At the

end of 24 hr the fish were removed and the water samples were analyzed for ammonia nitrogen, urea nitrogen, and pH.

Analytical Procedures

Total Nitrogen: Folin Farmer-Micro Kjeldahl Method

Water samples were analyzed for total nitrogen similar to the method outlined by Oser (1965). Digestion of the nitrogenous compounds to $(NH_4)_2SO_4$ and steam distillation of the ammonia was carried out in a Micro Kjeldahl Digestor Apparatus and a Micro Kjeldahl Distillation unit (Lab Con Co Corporation, Kansas City, Mo.). Color development proportional to the ammonia concentration was achieved by addition of Nesslers Compound, Koch-McMeekin Formula (Scientific Products, Evanston, Ill.) and spectro photometric analysis on a Spectronic 20 (Bausch and Lomb, Rochester, N.Y.) at 480 mμ. Bacterial action on the waste products is assumed to be negligible over the 24 hr experimental period (Gerking, 1955). For a detailed description of the analytical procedure, see Appendix I.

Ammonia Nitrogen: Permutit Method

The method used was that outlined by Oser (1965) for ammonia nitrogen determination by the Permutit method. It was

assumed that the volatility of ammonia was negligible (Gerking, 1955). See Appendix IIA for detailed description of the analytical procedure.

Urea Nitrogen:
Enzymatic Degradation

Water samples were placed in flasks containing 670 mg dry Permutit. One ml of Urease Glycerol Extract (Scientific Products, Evanston, Ill.) was added and the flask was shaken periodically for 30 min at room temperature, after which an analysis of ammonia formed was carried out in the same manner as for the ammonia nitrogen determination. The quantity of nitrogen analyzed is nitrogen from: (1) urea converted to ammonia, and (2) nitrogen initially present as ammonia. Urea nitrogen is then determined by subtracting ammonia nitrogen (from water samples analyzed for ammonia without enzyme degradation) from the total nitrogen existing as ammonia after enzymatic conversion. See Appendix IIB for analytical procedure.

Protein Nitrogen: Lowery Method

Protein nitrogen was determined by the method of Oyama and Eagle (1956). One ml of sample was incubated for 20 min with 5 ml of Lowery Reagent C. One-half ml of Folin and Ciocalteau

Phenol Reagent (Central Scientific Co., Chicago, Ill.) was added, and the resultant color developed was read on the Spectronic 20 at 660 mμ after 30 min.

pH

Determinations of pH were made at 20 C using a Beckman Expandomatic pH Meter (Beckman Instruments, Inc., Fullerton, Calif.). The instrument was calibrated with Standard Buffer Solutions, pH 7 and 10 (Sargent and Co., Detroit, Mich.).

Calculations

For all nitrogen assays the quantity of nitrogen in unknown samples (C_u) was calculated using the following equation (2):

$$C_u = \frac{C_s \times OD_u}{OD_s} \qquad (2)$$

Where: C_s = concentration of nitrogen in standard.

OD_s = optical density of standard.

OD_u = optical density of unknown.

Standard curves were made for all procedures to determine range and linearity of determinations. For each day's experiments a standard with a nitrogen concentration near that of the unknowns

was used, and calculations of the unknown concentrations were made using the OD of this standard. To minimize random error all standards and unknowns were run in duplicate and calculations made using the average OD of the two samples. Standard curves are given in Appendix III.

Gill Histology

The first and second pair of gill arches were cut from the fish and fixed in formaldehyde, acetic acid and alcohol (FAA)solution for 48 hr. Tissues were then dehydrated and cleaned in Tetrahydrofuran (Fischer Scientific Company, Fairlawn, New Jersey), vacuum embedded in Paraplast (Arthur H. Thomas Co., Philadelphia, Penn.), and sectioned at 8 μ. Sections were stained using the Hematoxylin and Eosin as described in the Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology (Luna, 1968).

RESULTS

In the ensuing discussion the word ammonia which appears in either the text or in tables and figures will refer to total ammonia, i.e., $NH_3 + NH_4^+$. During the course of the experiments with different concentrations of ambient ammonia the pH of the environmental water increased by as little as 0.10 and as much as 0.39 of a pH unit. According to the Henderson-Hasselbalch equation ($pH = pK + \log NH_3/NH_4^+$), the increase in pH gives rise to an increase in the ratio NH_3/NH_4^+.

Experiments With Trout

When rainbow trout were subjected to increased levels of ambient ammonia, the total amount of nitrogen excreted decreased and the values obtained at mean concentrations of 6.05 and 8.30 μg ammonia/ml were significantly lower than the value at 2.39 ($p = 0.001$) The data presented in Figure 1 indicate that at the two high ammonia levels the total nitrogen excreted tends to approach a constant rate. In all determinations of significance the Student t-test was used and an alpha level of 10% was considered to be significant.

Figure 1. --The effect of 24 hr exposure to various ambient ammonia concentrations at 13 C on total nitrogen excretion by rainbow trout. Plotted are mean ± standard error of (n) observations.

Figure 2. --The effect of 24 hr exposure to various ambient ammonia concentrations at 13 C on ammonia nitrogen excretion by rainbow trout. Plotted are mean ± standard error of (n) observations.

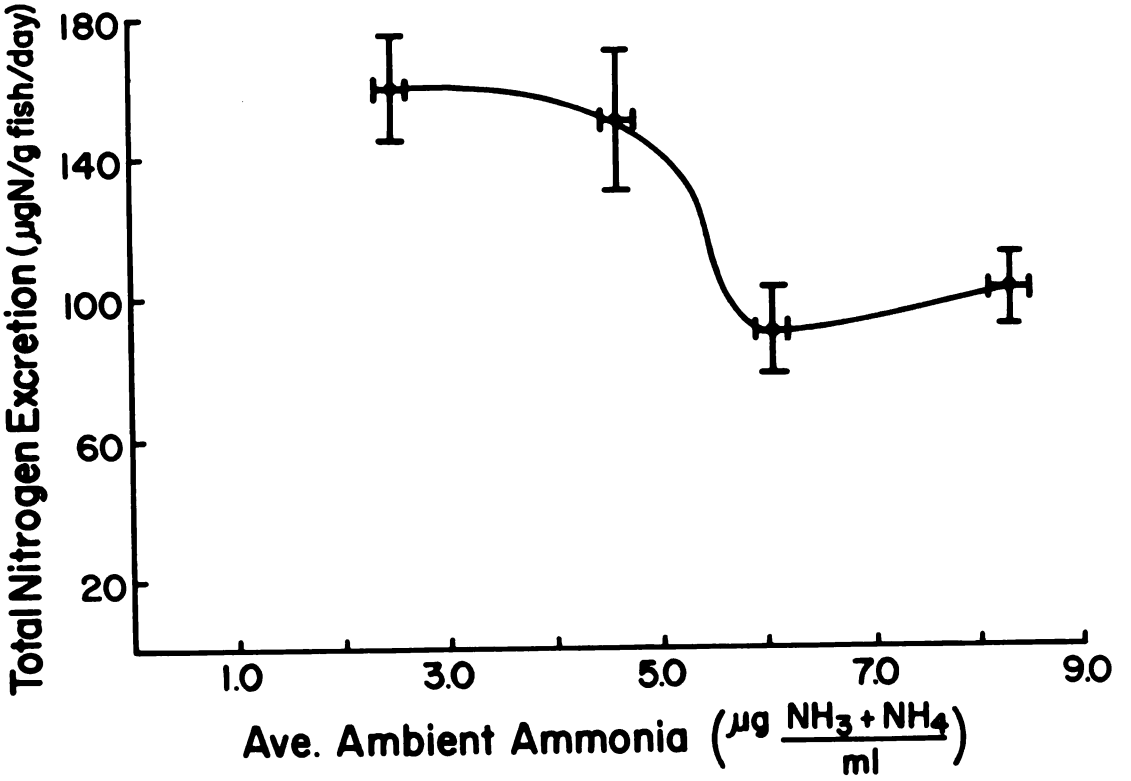

Figure 1

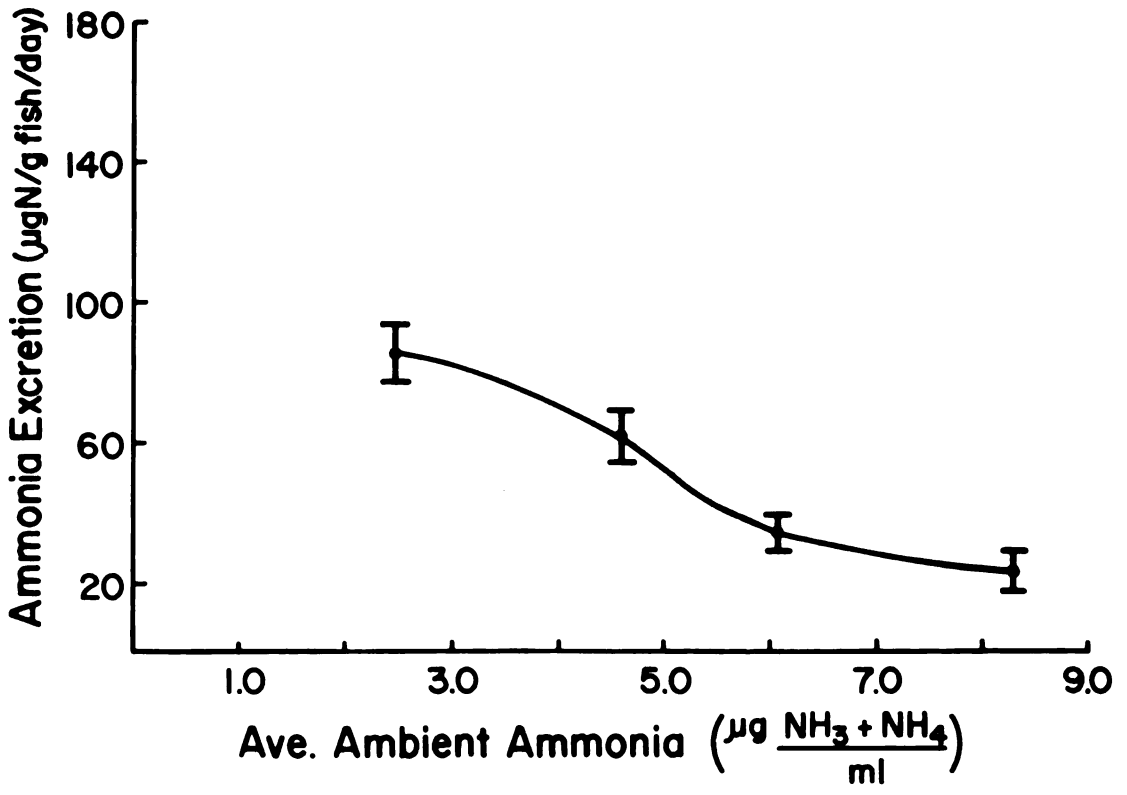

Figure 2

Figure 3. -- The effect of 24 hr exposure to various ambient ammonia concentrations at 13 C on urea nitrogen excretion by rainbow trout. Plotted are mean ± standard error of (n) observations.

Figure 4. -- The effect of 24 hr exposure to various ambient ammona concentrations at 13 C on protein nitrogen excretion by rainbow trout. Plotted are mean ± standard error of (n) observations.

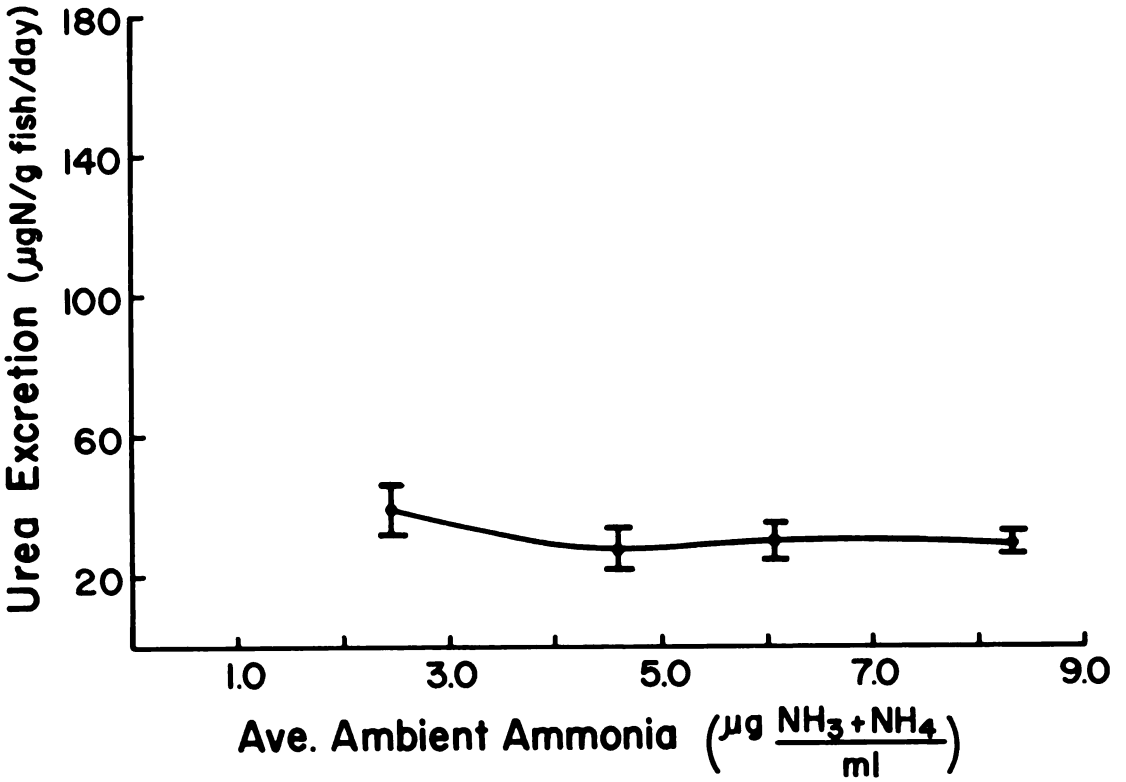

Figure 3

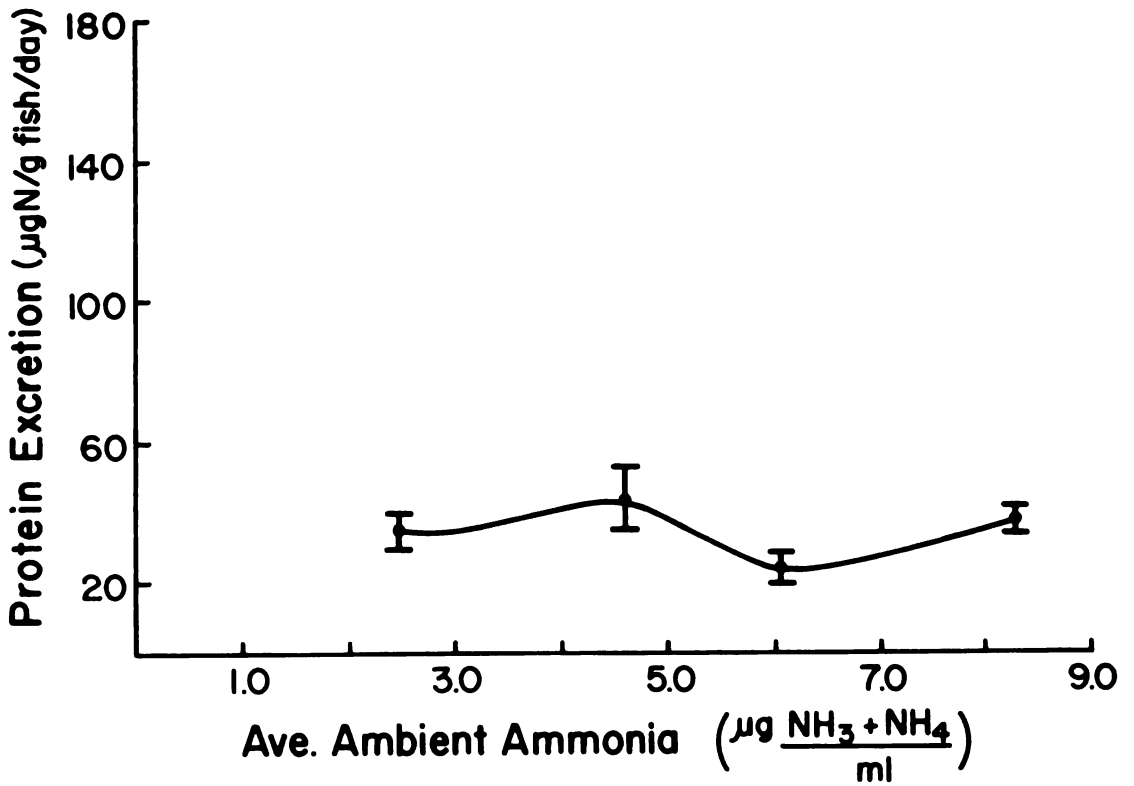

Figure 4

Concomitant with the decrease in total nitrogen excreted there was a significant ($p = 0.001$) decrease in the amount of nitrogen excreted as ammonia (Figure 2). As with the values for total nitrogen excretion, the ammonia excretion values appear to level off at the high ambient ammonia concentrations. No significant change in the amount of nitrogen excreted as urea or protein was noted over the range of ammonia exposures tested (Figures 3 and 4). Data for all of the above experiments is summarized in Table 1. The percent nitrogen accounted for was determined by using the following equation (3):

$$\% \text{ accounted for} = \frac{\text{nitrogen excreted as ammonia, urea and protein}}{\text{total nitrogen excreted}} \times 100 \qquad (3)$$

In a separate and somewhat similar study on trout subjected to very low levels of ambient ammonia (0.45 μg/ml for 24 hr) excreted significantly less ($p = 0.001$) urea than when they were subsequently exposed to high ambient ammonia (5.11 μg/ml) for nearly the same time period (Table 2). At the high ammonia level, five of the seven trout died several hours prior to the end of the 24 hr experimental period. It is assumed that if the fish had lived the full 24 hr the amount of urea excreted would have been even greater.

Table 1. --Excretory nitrogen components from rainbow trout subjected to various levels of ambient ammonia. Values for nitrogen excretion are as μg N/g of fish per day. The % N accounted for is excreted ammonia N + urea N + protein N × 100/total N excreted

Average ambient ammonia (μg/ml)	Ammonia N excretion	Urea N excretion	Protein N excretion	Total N excretion	% N accounted for
2.39 ± 0.07 (19)*	86 ± 7.8	39 ± 7.6	35 ± 4.9	161 ± 15.9	99
4.50 ± 0.17 (12)	63 ± 7.2	28 ± 5.8	44 ± 9.0	151 ± 19.9	89
6.05 ± 0.13 (10)	35 ± 4.5	30 ± 4.9	24 ± 4.3	91 ± 11.0	97
8.30 ± 0.21 (17)	24 ± 6.9	30 ± 3.4	38 ± 4.2	103 ± 10.1	89

*mean ± standard error (n)

Table 2. --Effect of ambient ammonia on urea excreted by rainbow trout. See text for details.

Run	Average ambient ammonia (μg/ml)	n	Urea excretion as μg N/g of fish per day
1	0.45 ± 0.04*	7	12 ± 1.8
2	5.11 ± 0.75	7	43 ± 5.0

*mean ± standard error

To test the possible effects of acclimation to increased ambient ammonia on urea excretion, two groups of trout were subjected to 5.00 μg ammonia/ml and to less than 0.50 μg/ml for 40 days. The rate of urea excretion by each group was measured while they were exposed to an average ambient ammonia concentration which ranged from 3.0 to 3.5 μg/ml (Table 3).

Table 3. --Effect of exposure to high levels of ammonia for 40 days on urea excretion by trout. Urea excretion was measured over a 24 hr period

Group	Average ambient ammonia during acclimation (μg/ml)	n	Urea excretion as μg N/gm of fish per day
1	0.50	6	23 ± 4.8*
2	5.00	6	13 ± 3.5

*mean ± standard error

The data indicate that there was less urea excreted by trout adapted to the higher ammonia and the difference is statistically significant.

Histological Observations

Gills of trout exposed to low (0.5 μg/ml) ammonia (Figure 5) have long, slender lamellae which exhibit no significant pathology. The gill lamellae from trout exposed to high ambient ammonia (5 μg/ml) are shorter and thicker with bulbous ends (Figure 6). Some consolidation of lamellae was also noted in fish exposed to high ammonia. In the higher power photomictographs of gill lamellae from trout exposed to high ambient ammonia two types of pathology can be seen. Many filaments show a rather limited hyperplasia (Figure 7) which is accompanied by the appearance of cells containing large vacuoles whose contents stain positive for protein. Other lamellae (Figure 8) show a definite hyperplasia of the epithelial layer as is evident by an increase in the number of cell nuclei. Gills of goldfish exposed to 25.0 μg ammonia/ml for 6 weeks exhibited no significant pathology when compared to gills of goldfish exposed to less than 0.50 μg ammonia/ml for the same time period.

Figure 5. --Photomicrograph of gill lamellae from rainbow trout exposed to low ammonia (< 0.5 μg ammonia/ml) concentrations for 8 weeks at 13 C. The ambient ammonia concentration was maintained at the lowest possible level to minimize ammonia effects on the gills. H & E × 133.

Figure 6. --Photomicrograph of gill lamellae from rainbow trout exposed to 5 μg ammonia/ml for 8 weeks. Note the short and thick lamellae with enlarged terminal ends.

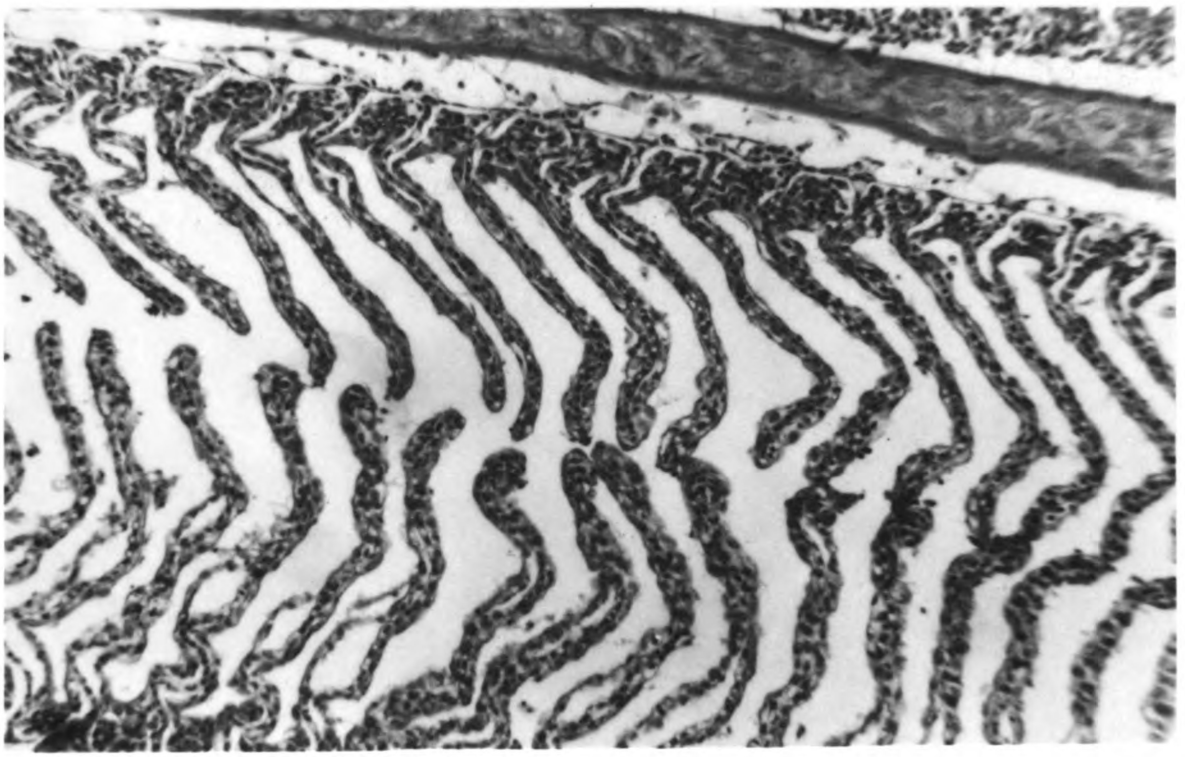

Figure 5

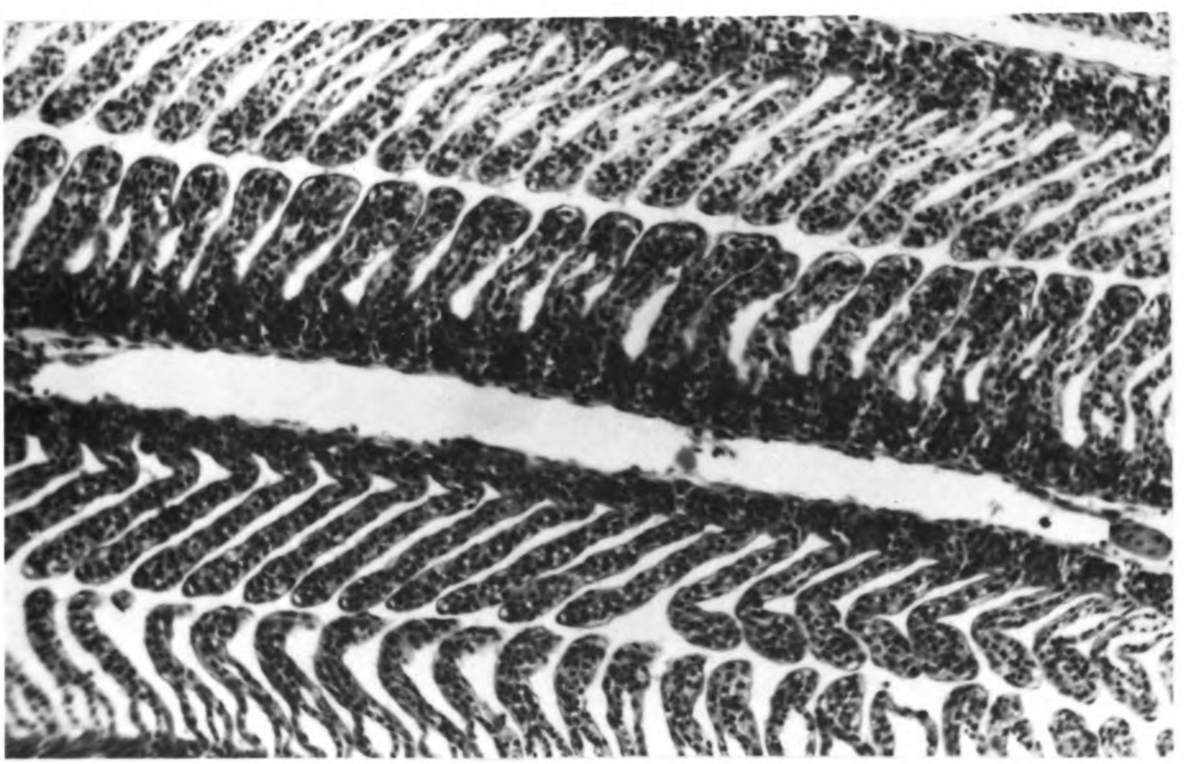

Figure 6

Figure 7. --Lamellae from trout exposed to 5 μg ammonia/ml for 8 weeks. Note the vacuolization of the epithelial cells and very limited hyperplasia. H & E × 533.

Figure 8. --Lamellae from trout exposed to 5 μg ammonia/ml for 8 weeks. Definite hyperplasia of the epithelial cells is noted. H & E × 533.

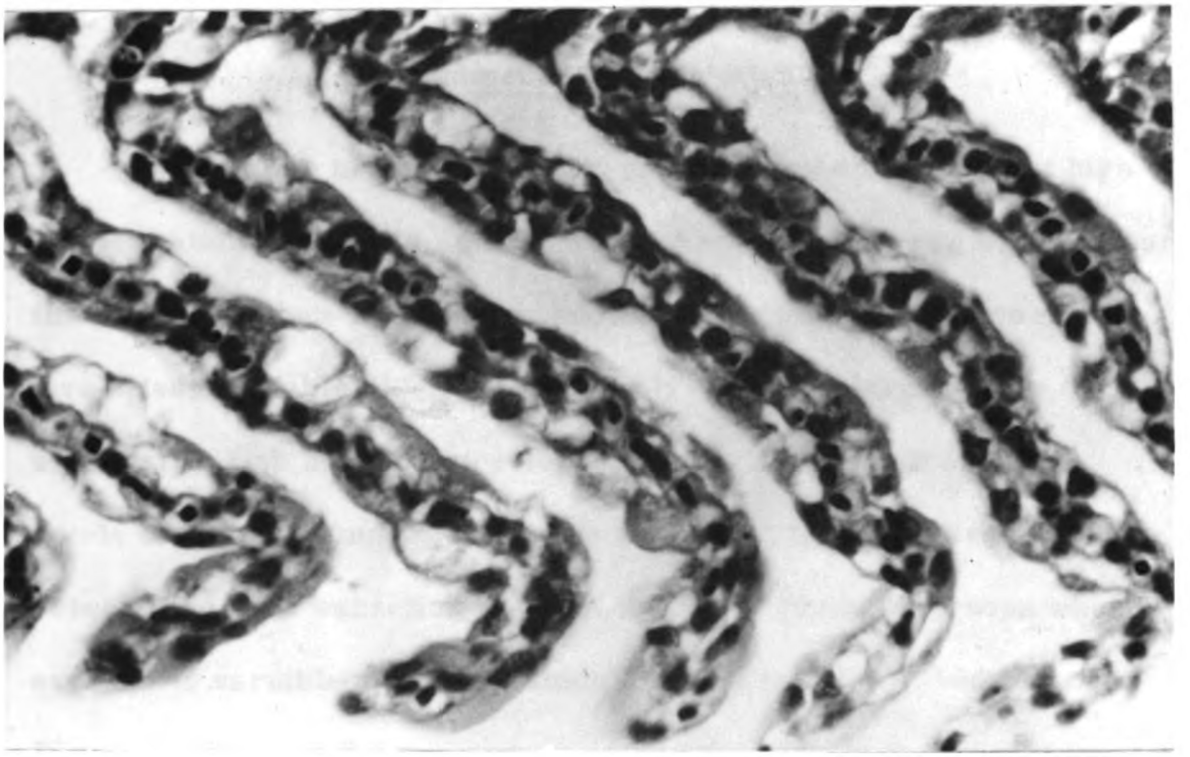

Figure 7

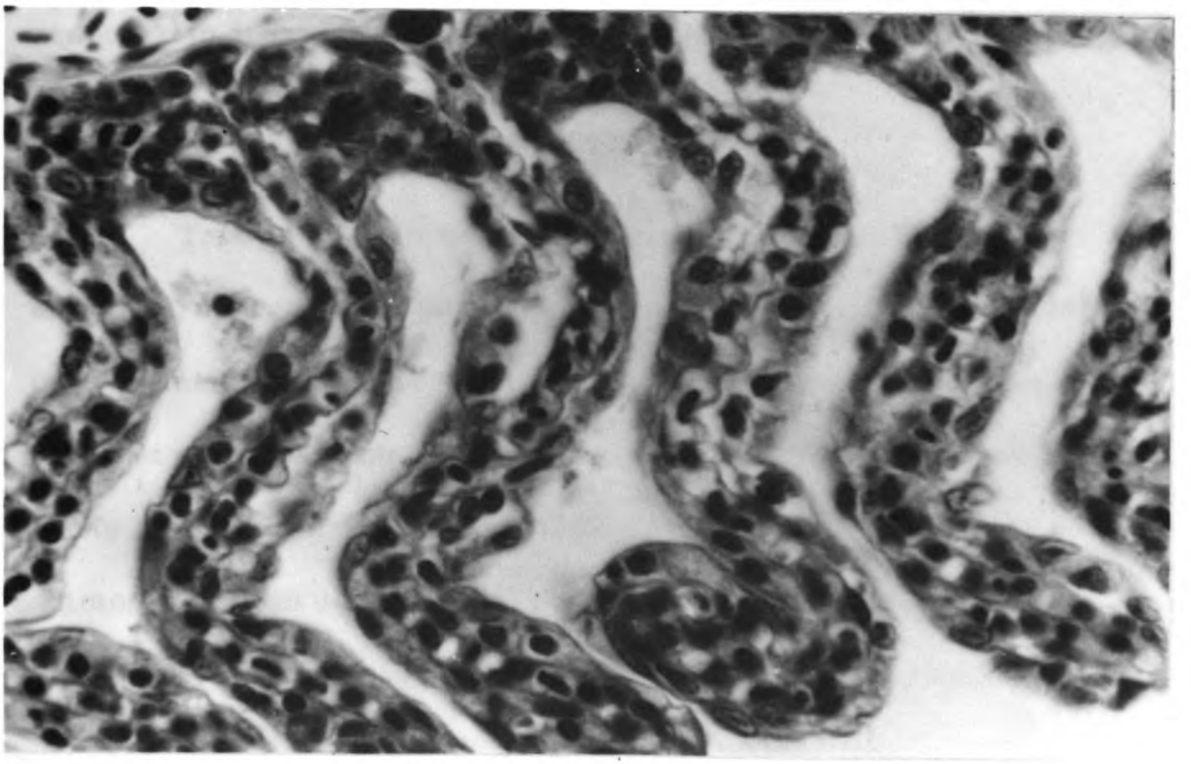

Figure 8

Experiments With Goldfish

When the urea excretion of goldfish was determined at high ambient ammonia levels, the urea nitrogen excretion was at least four times greater than when determinations were made at the lowest levels tested (Table 4). This invariably occurred irrespective of whether the fish had been previously acclimated to high or low levels of ambient ammonia. There was no statistical difference between the urea excretion rates of the two acclimated groups when exposed to variable ambient ammonia levels for 24 hrs, and in Figure 9, data for the two groups are combined. In terms of urea nitrogen excretion, apparently the response to high ambient ammonia is very rapid in goldfish and trout, but the degree of response in trout is of a much lower magnitude.

Qualitative Observations

Trout placed in water containing more than 3.0 μg ammonia/ml became hyperexcitable. Any disturbance of the tank or movements above the tank visible to the fish resulted in dis-oriented escape attempts which sent the fish crashing into the sides of the tank. If these fish were then placed in water containing no ammonia, within several days they appeared to return to normal, i.e., were no longer hyperexcitable. The highest ammonia

Table 4. --Urea excretion during 24 hrs at various levels of ambient ammonia by goldfish which had been acclimated to either low (0.5 μg/ml) or high (5.0 to 25.0 μg/ml) ambient ammonia

Days fish acclimated	Initial acclimation concentration μg ammonia/ml	n**	Ambient concentration during experiment μg ammonia/ml	Urea excretion μg N/g of fish per day
26	0.5	7	2.37 + 0.23*	134 + 31.0
20	0.5	3	0.75 + 0.03	52 + 6.8
30	0.5	7	0.08 + 0.01	27 + 2.9
26	5.0	11	2.22 + 0.12	128 + 21.0
20	25.0	4	0.68 + 0.11	22 + 10.1
30	25.0	9	0.10 + 0.01	28 + 1.8

*mean ± standard error

**n equals the number of containers of goldfish.

Figure 9. --The effect of 24 hr exposure to various ambient ammonia concentrations at 20 C on urea excretion in goldfish.

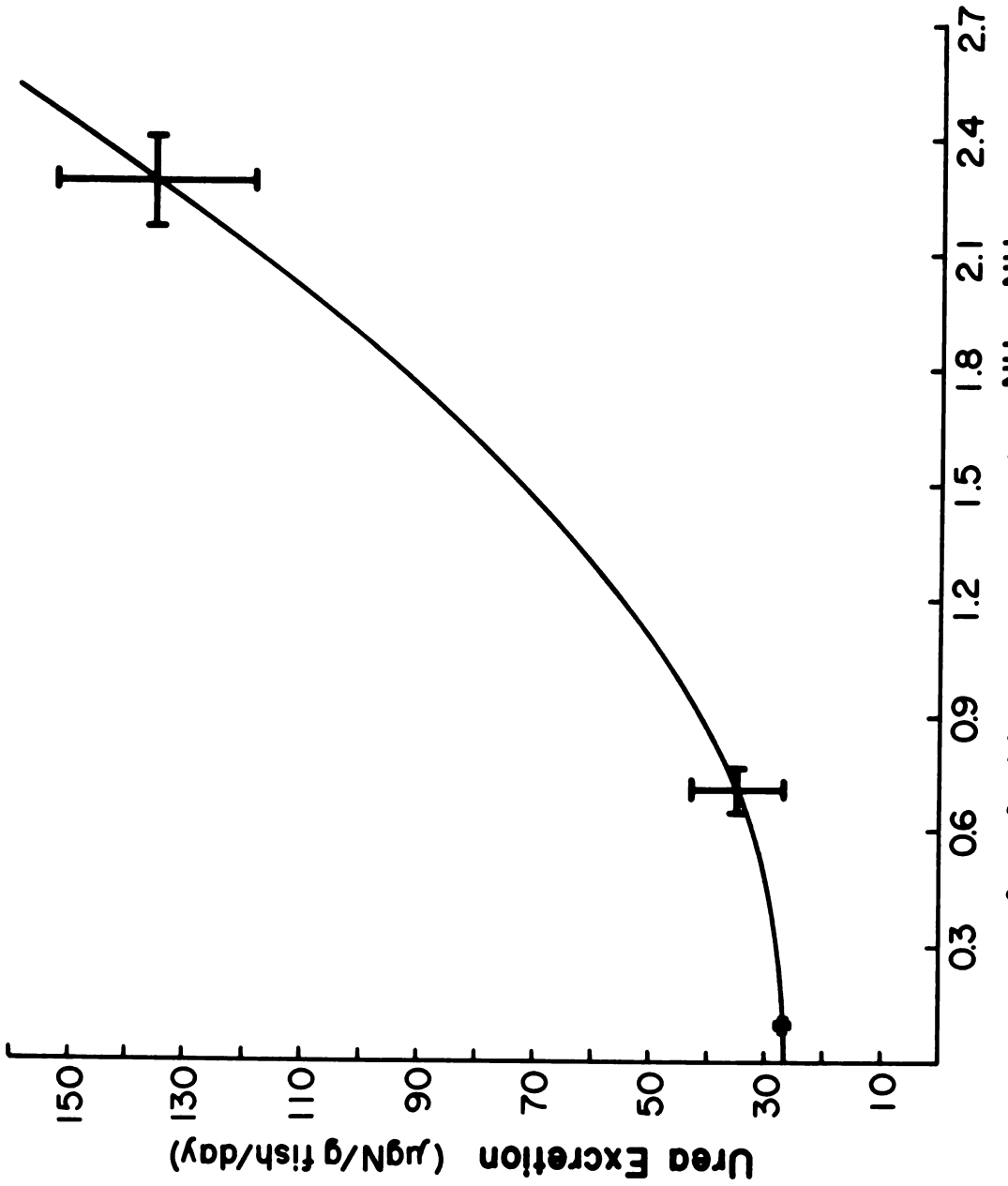
Urea Excretion (μgN/g fish/day)
150
130
110
90
70
50
30
10
0.3
0.6
0.9
1.2
1.5
1.8
2.1
2.4
2.7
Ave. Ambient Ammonia (μg NH3 + NH4 / ml)

Figure 9

concentration (8.30 μg ammonia/ml) to which trout were exposed caused about 50% mortality within 24 hrs. There was a decrease in mortality with the corresponding decrease of ambient ammonia concentrations. The onset of death was characterized by violent thrashing movements which were functionless as propulsive swimming movements.

Trout used in the high ammonia acclimation experiments were also hyperexcitable initially; however, after about two days they appeared to calm down, and after the third day they showed no signs of elevated excitability. Conversely, goldfish did not appear to be bothered at all by ammonia concentrations of as high as 25.0 μg/ml, eight times greater than that which affected the trout. When the goldfish were placed in 40.0 μg ammonia/ml, about 10% died in 24 hrs. The onset of death was characterized by a gradual cessation of swimming movements, during which time the fish slowly settled to the bottom of the aquarium. After 1 or 2 hrs nearly all of the dying fish curled laterally in the form of a "U" and opercular movements dropped considerably. If left in the ammonia solution, death soon followed. Three fish near death were removed from the ammonia water and placed in ammonia-free water; two of the fish lived for several days before dying and the third completely recovered. Although severe, the effects of ammonia on fish apparently are, to some extent, reversible.

DISCUSSION

Nitrogen Excretion Patterns in Rainbow Trout

Rainbow trout subjected to increasing concentrations of ambient ammonia excreted decreasing amounts of total nitrogen (Figure 1). At higher levels of ambient ammonia the total nitrogen excretion appeared to reach a steady rate. Nitrogen excreted as ammonia exhibited a pattern similar to total nitrogen excretion, i.e., an initial decrease with a leveling effect at higher ambient ammonia concentrations. Nitrogen excreted as urea and protein remained relatively constant under all experimental conditions.

The change in total nitrogen excretion reflects a change in ammonia nitrogen excretion. Changes in the rate of both total and ammonia nitrogen excretion occurred at the same concentration of ambient ammonia. Fromm and Gillette (1968) reported that the percent of total nitrogen excreted as ammonia nitrogen decreased from 52% to 29% when trout were subjected to increased ambient ammonia concentrations. They postulated that the decrease in percentage of ammonia nitrogen excreted was compensated by an increase in

excretion of some other nitrogenous compound. To state categorically that there was an increase in the excretion of nitrogen containing compounds other than ammonia, however, could depend on interpretation of the data. Data from the present experiment (Table 5) also show a similar decreased percentage of total nitrogen excreted as ammonia (53% and 26% for low and high ambient ammonia levels, respectively) and an increased % excretion rates for urea and protein nitrogen (21% to 34% and 21% to 36%, respectively, as ambient ammonia was elevated).

Table 5. --Components of nitrogen excretion as percent of total nitrogen excreted by rainbow trout subjected to various average ambient ammonia levels. Refer to text for discussion.

Average ambient ammonia	% of total nitrogen excreted		
	Ammonia nitrogen	Urea nitrogen	Protein nitrogen
2.39 + 0.09 (19)*	53	21	21
4.58 + 0.17 (12)	40	25	28
6.05 + 0.13 (10)	40	32	27
8.30 + 0.21 (17)	26	34	36

*mean ± standard error of (n) observations

Actual values (Table 1) for nitrogen excretion (μg N/g of fish per day), however, demonstrate no significant increase in either protein or urea nitrogen excretion; therefore the increased percentage of urea and protein is due to a decreased amount of total nitrogen excreted.

Ammonia excretion across the gill can occur by two processes, an active transport "pump" of NH_4^+ coupled with sodium uptake or passive diffusion of NH_3. Maetz and Romeu (1964) demonstrated an active transport of sodium ion into the fish (Carassius auratus) in exchange for ammonium ions, and under resting conditions, the net flux of sodium uptake was of the same order of magnitude as the ammonia excreted. They also reported that the addition of ammonium ions to the external medium inhibits net sodium uptake (and presumably ammonium ion transported excretion), while addition of ammonium ions to the blood enhances net sodium uptake (and presumably ammonium excretion). Rainbow trout placed in ambient ammonia concentrations of from 0 to 8 μg/ml had blood ammonia values increase from 39 to 70 μg/ml (Fromm and Gillette, 1968). The greater increase of blood ammonia as opposed to the increase in total environmental ammonia would suggest an overall enhancement of the "pumping mechanism" and an increased removal of ammonium from the fish. The results of the

present experiment and those of Fromm and Gillette (1968) show that the excretion of ammonia ($NH_3 + NH_4^+$) decreased with elevated blood ammonia. This would indicate that the "pump" either becomes less efficient due to elevated blood sodium levels or is saturated with substrate (NH_4^+) at lower blood ammonia levels, and at high blood levels another mechanism of ammonia excretion such as diffusion becomes predominant.

Weak acids and bases diffuse mainly when in the nonionized lipid soluble form (Jacobs, 1940), and therefore the diffusion of ammonia or ammonium would be as NH_3. The blood-environmental water ammonia gradient (expressed as μg ($NH_3 + NH_4^+$)/ml) was 40 to 0 for low ambient ammonia and 70 to 8 for high ammonia; however, the blood-environmental water gradient for unionized ammonia (expressed as μg NH_3/ml) was 0.6 to 0.0 at low ambient ammonia levels and 1.13 to 0.8 at high levels (Fromm and Gillette, 1968). As ambient ammonia is increased, the blood-environmental water diffusion gradient for total ammonia ($NH_3 + NH_4^+$) increased, but the gradient for diffusable ammonia (NH_3) decreased. The decrease in the diffusion gradient for NH_3 could account for the decrease in ammonia excretion by the fish at higher ambient ammonia levels (see Figure 2).

The results indicate that extended periods of exposure (8 weeks) to ammonia cause histological changes to occur in the gill lamellae. Burrows (1964) found similar changes in the lamellae of gills of chinook salmon (Oncorhynchus tshawylscha) after 6 weeks exposure to 0.7 ppm NH_4^+ (approximately 0.7 μg/ml). A one week exposure to 5 μg NH_4^+/ml (Reichenbach-Klinke, 1968) caused the typical histopathology in rainbow trout including the gills. It is possible that, in the present experiment, exposure to the various ammonia concentrations for 24 hr could result in a change in the permeability of the epithelial cells of the lamellae which could hinder the diffusion of NH_3 and/or the active exchange transport system of NH_4^+.

The lack of any consistent change in the rate of urea excretion at the elevated ammonia levels examined indicates that the urea excretion in rainbow trout is independent of the environmental ammonia level, especially at concentrations above 1 μg/ml. Data presented on blood ammonia values by Fromm and Gillette (1968) would also indicate that there is no correlation between the level of blood ammonia and the amount of urea excreted by rainbow trout.

Protein excretion (Figure 4) at the various ammonia levels appears to fluctuate, but there is no general trend either to increased or decreased excretion following a 24 hr exposure to ammonia. The

source of protein excreted is probably mucoprotein, and it was found that mucus scraped from the epidermis of fish had a high nitrogen content. Jones (1964) summarized information on the effects of pollutants on mucus secretion and stated that there appears to be a great species specificity with regard to the effect of heavy metals and fine particulate matter on the quantity of mucus secreted by fish. Generally an increase in either the concentration of heavy metals or particulate matter causes increased mucus secretion. Jones also gave evidence that mucus may affect the permeability of the integument to salts or other compounds. In the present experiment, it is probable that the ambient ammonia acts as an irritant to the fish and causes increased mucus secretion. If the mucus secreted by the gills remains associated with the gills rather than being sloughed off, it is conceivable that ammonia excretion could be hindered and increased levels of mucoprotein could not be detected in the environmental water.

In characterizing the components of nitrogen excretion, from as little as one to as high as eleven per cent of total nitrogen excreted could not be accounted for as ammonia, urea, or protein. The variation between values for total nitrogen by the Micro Kjeldahl method and the sum of ammonia, urea, and protein nitrogens might be due to the combined errors in the four analytical procedures,

but because the total nitrogen by the Micro Kjeldahl method is consistently greater than the sum of nitrogen determined by the other three methods, it seems more probable that another nitrogenous compound is being excreted by the fish. Smith (1929) reported that urinary creatine could contribute up to eight per cent of the total nitrogen excreted by goldfish or carp (Cyprinus carpio), and there also exists an excretory amine or amine oxide. Irrespective of the inability to account for the unknown compound, the percentage of the unknown excretion compared to total nitrogen excreted appears not to increase with increasing ambient ammonia concentrations.

No analyses were made of protein, ammonia, and total nitrogen excretion from the seven trout subjected to very low ammonia levels (0.4 μg/ml). As urea excretion decreased at this ammonia level it would be interesting to know if any other parameter of nitrogen excretion changed also.

Urea Excretion in Rainbow Trout and Goldfish

Rainbow trout and goldfish acclimated to elevated ammonia levels for extended periods of time, when compared to fish subjected to very low ammonia levels for the same time period, exhibited no increase in urea excretion when both were placed in similar ammonia levels for 24 hrs. The acclimated trout, in fact, decreased

the amount of urea excreted over the 24 hr period. Failure to show any increase in urea excretion could be due to any of three possible mechanisms: (1) the change in urea excretion could be so rapid that fish acclimated to high and low ammonia levels, when placed in the same concentration of ammonia, would show no significant difference in the quantity of urea excreted; (2) urea excretion is not dependent on blood ammonia levels; and (3) the functional enzyme complement for urea synthesis is not present.

When goldfish, acclimated to low ammonia levels, were placed in high ambient ammonia concentrations for 24 hrs, they excreted urea at the same rate as did the goldfish acclimated to high ammonia levels. Conversely, goldfish acclimated to high ambient ammonia concentrations and placed in very low ambient ammonia for 24 hrs excreted urea at a rate identical to the fish acclimated to low ambient ammonia. The amount of urea excreted was dependent on the 24 hr experimental bath ammonia concentrations and not related to acclimation ammonia concentration or duration of acclimation. The ability of the goldfish to change the urea excretion rate concomitant with the change in ambient ammonia appears to be either instantaneous or with a time course so short that any lag time is insignificant in 24 hrs.

The change of urea excretion with changing ambient ammonia levels could possibly be explained by activity of two urea synthesizing pathways, the ornithine cycle or the purine pathway. The ornithine cycle incorporates two ammonia molecules and carbon dioxide in the synthesis of urea. Of the three end products of the cycle, urea, fumarate, and ornithine, the latter two can be reused in further urea synthesis. Four ATP's are required in the synthesis and, other than the two ammonia molecules and one carbon dioxide molecule, no other compounds are lost by the organism, making the cycle relatively efficient. The synthesis of purines is a very complex process in which there is not only energy utilized in the synthetic steps, but the utilization of glucose as ribose deprives the animal of energy that could have been derived through glycolysis and Krebs cycle activity. Amino acids such as glycine or serine are also needed as they are incorporated in the purine molecule. The relative importance of either pathway depends on the concentrations and activity of the enzymes involved and/or on the ability of the organism to synthesize the enzymes of either pathway to levels which make them functionally useful.

Ornithine Cycle

The absence of two ornithine cycle enzymes in the three teleosts studied, perch (Perca flavescens), carp (Cyprinus carpio),

and brown trout (Salmo trutta fario) (Brown and Cohen, 1960) led the authors to believe that the genetic complement of these enzymes had been deleted prior to the evolution of the teleostei. (The term deletion was interpreted by the authors to mean the lowering of the enzymatic activity to a level at which the urea cycle no longer plays a significant role in the disposal of ammonia as urea). The authors also stated that it is not known if the loss of activity can be due to loss of the primary genetic information responsible for enzyme synthesis.

More recently Huggins et al. (1969) provided evidence that all ornithine cycle enzymes are present in many teleosts, but at low levels of activity. Assuming the enzyme with the lowest moles of product formed/hour per gram of liver to be the rate limiting enzyme, the maximum urea synthesis through ornithine cycle activity by rainbow trout, according to Huggins et al., would be 0.13 μ moles urea/hr per g wet wt liver. If the liver of trout is 1.02% of body weight (Schiffman, 1957), the maximum urea synthesis would be 0.63 μg N/g fish/day. In the present study, in which around 30 μg N/g fish/day were formed, the amount of urea nitrogen resulting from the enzyme activity reported by Huggins et al. would be insignificant.

Huggins et al. also found that activity of one of the enzymes of the ornithine cycle (arginiosuccinate lyase) is below levels of detection in goldfish. All enzyme assays by these researchers were carried out at optimum conditions for each enzyme (pH 7 to pH 9.5 and 37 C), and the enzymes are probably less efficient catalytically in vivo than indicated. If the ornithine cycle functioned in the two teleosts studied in the present experiment, either the enzyme assays by Huggins et al. are not valid for an in vivo system or the enzymes must have been synthesized de novo by an enzyme inducing system.

The process of enzyme induction is characterized by an increase in the total amount of enzyme which gives rise to an increase in the amount of catalytic action (Filner, Wray, and Varner, 1969). Recent literature has been reviewed by Conney (1967) and it is known that certain agents, "inducers," when present in critical amounts can cause a de novo synthesis of many enzymes. This ultimately results in substrate utilization and product formation rates up to tenfold greater than normal.

An inherent characteristic of induction is a noticeable time lag from addition of the inducer to synthesis of the enzyme proteins. In rats the induction process can take from 3 hrs to 10 or more days, depending on the specific enzyme induced and the concentration of the inducer (Conney, 1967). It is believed that in higher animals control

of enzyme induction or of protein synthesis in general is based on genetic regulation carried out in a minimum of five steps (Britten and Davidson, 1969). The sequence steps necessary in altering protein synthesis are: (1) response to an external signal, (2) production of a second signal, (3) transmission of a second signal to a number of receptors unresponsive to the original signal, (4) reception of the second signal, and (5) response to this event by activation of a producer gene and its transcription providing the cell with the producer gene product. The producer gene is that sequence on the DNA molecule which codes for the RNA directly responsible in protein synthesis. Britten and Davidson implicate this form of genetic regulation in the urea cycle enzymes and such may be the case for a metamorphosing amphibian which shows a gradual increase in the enzyme levels (Brown et al., 1959). Regulatory steps require synthesis of intermediates, and it seems probable that the time factor would be inconsistent with the apparent instantaneous change in urea excretion rates found in the present study with teleosts.

Another form of "enzyme induction" is characterized by a decrease in the degradation rate of an enzyme. It has been demonstrated that the amount of liver arginase in the rat can be increased by decreasing the rate of arginase degradation (Conney, 1967);

however, arginase has a half life of 4 to 5 days. If this mechanism plays a role in the urea synthesis by the ornithine cycle, there must be a regulatory enzyme with an extremely short half life to account for the rapid change in urea excretory rates.

Purine Catabolism

Activities of enzymes involved in purine degradation (urate to urea) were assayed for in various species of marine teleosts (Goldstein and Forster, 1965) and found to range from 23.2 μ moles urea produced/g-liver per hr in the winter flounder (Pseudopleuronectes americanus) to 5.2 μ moles urea produced/g-liver per hr in the American goosefish (Lophius americanus). If the urea production found is calculated in terms of μg N/g fish per day, the resulting range is 155 to 35 μg N/g fish per day. Optimum conditions were used for the enzyme assays (pH 7.4, 25 C) and the fact that all fish used by Goldstein and Forster were marine species might invalidate a comparison to fresh water teleosts; however, the urea excretion rates which they reported are of the same order of magnitude as that found in the present study for fresh water teleosts.

Cvancara (1969) assayed liver uricase for a variety of fresh water teleosts, but the data is reported as specific activity (units/mg protein) and it was not possible to convert his data to

μg N/g fish per day for purposes of comparison. He reported in passing that values for uricase and probably allantoinase and allantoicase were of the same order of magnitude as those found in marine teleosts by Goldstein and Forster (1965). The activities of the purine degradory enzymes may be sufficiently high to account for most if not all of the urea excreted by the two teleosts in the present study. The ability of goldfish to excrete more urea than trout probably reflects the relative enzyme concentrations present in the two species.

As the experimental animals were kept at two different temperatures, the species variation in urea excretion rates could possibly be due to the effect of temperature on the enzyme kinetics. It is believed, however, that the urea excretion rates are reflective of a species difference. Hochachka and Somero (1968) have found that in poikilotherms the enzyme lactic dehydrogenase adapts to the temperature of the organism such that, regardless of the temperature the organism is acclimated to, the enzyme functions maximally. In all probability, other enzyme systems act similarly.

Although there are many questions left unanswered in these experiments, there are two basic areas of research which bear further investigation.

(1) Comparative enzymology of urea producing enzymes:

Is the apparent species difference in ammonia associated urea production consistent with an overall evolutionary or ecological position of various teleost species? Is the primary pathway for urea production ornithine cycle activity, purine synthesis or some other little-known pathway? How does ammonia regulate urea synthesis, i.e., does it increase effective substrate levels or merely act as an enzyme activator?

(2) Molecular exchange across the gill:

Is the mechanism of ammonia excretion via the gill passive diffusion, active transport or a combination of both? As ambient ammonia increases, ammonia excretion decreases. Does this decrease reflect failure of either passive diffusion or active transport of ammonia? How does increased blood ammonia affect blood pH; and if blood pH changes, how could this affect an ammonia excretion mechanism?

SUMMARY AND CONCLUSIONS

Rainbow Trout

1. When rainbow trout were exposed to ammonia concentrations ranging from about 2 to 8 μg/ml for 24 hr at 13 C the total nitrogen excreted decreased from an average of 161 to 103 μg N/g fish per day. The decreased total nitrogen excretion rate reflects a decrease in excretion of ammonia from 86 to 24 μg ammonia N/g fish per day. Except for an initial increase in urea excretion at very low ammonia levels, the urea and protein excretion rates remained relatively constant at all other ambient ammonia levels.

2. Nearly all, around 94%, of the total nitrogen excreted by trout consisted of ammonia, urea and protein nitrogen.

3. Rainbow trout acclimated to 5 μg ammonia/ml showed no increase in urea excretion when compared to trout acclimated to as little as 0.5 μg ammonia/ml.

Goldfish

1. When goldfish were subjected to ambient ammonia ranging from 0.09 to 2.28 μg/ml the rate of urea excretion increased

from 27 to 136 μg N/g fish per day.

2. Acclimated goldfish exposed to various ambient ammonia levels for 24 hr demonstrated that the rate of urea excretion is dependent upon the ambient ammonia levels which prevail during the 24 hr experiment and are independent of both the acclimation ammonia concentrations and duration of acclimation.

3. The time course for change in urea excretion rates with the change in ambient ammonia concentrations is nearly instantaneous.

Species Variability Between Rainbow Trout and Goldfish

1. When exposed to similar ambient ammonia levels, goldfish excrete about three times more urea than trout, and urea excretion rates are much more responsive to ambient ammonia levels in goldfish.

2. Goldfish are able to survive ammonia levels more than three times greater than that which is lethal to trout (8 μg ammonia/L).

3. Hyperexcitability due to ammonia exposure which was observed in trout was not noticeable in goldfish.

4. There appeared to be greater histopathology of the gills of trout exposed to ammonia than to the gills of goldfish exposed to the same or greater ammonia concentrations.

5. Enzyme studies by other investigators support the hypothesis that purine catabolism is probably the main pathway for urea synthesis at the rates found in the present experiment.

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APPENDICES

APPENDIX I

PROCEDURE FOR ANALYSIS OF TOTAL NITROGEN

A. Digestion

1. To each 30 ml Digestion Flask (Lab Con Co*), add 1 ml of 50% (v/v) H_2SO_4 and one small glass bead.
2. Add 25 ml of sample to each flask, make duplicate determinations of all samples.
3. Set burner temperature to hottest setting.
4. Place sample on burner under a hood to remove fumes.
5. When white fumes form, add approximately 10 drops of 30% commercial H_2O_2 (add slowly to prevent spattering, keep under hood).
6. Keep flasks on moderate heat for 5 min. Then cool to room temperature.

B. Distillation

1. Start steam generator as in instruction manual.

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*Lab Con Co Instruction Manual, Parts List and Accessories, Laboratory Construction Company, Kansas City, Mo.

2. Pour digest from cooled flasks into addition funnel on distillation apparatus. Add digest to inner chamber, followed with 3 washings of flask and bead with ammonia-free water. Use as little water as possible for the washings.
3. Add 2 ml of 0.1 N H_2SO_4 to a 25 ml erlenmeyer flask and place flask under condenser such that the tip of the condenser is completely submerged in the acid and the gas inside the condenser cannot contact room air without passing through acid water seal.
4. Add 5 ml of 40% NaOH (w/v) to addition funnel, then add this slowly into the inner chamber. If the NaOH is added too fast, the reaction will be violent and ammonia will be lost in large bubbles blown through the acid water or the acid water will be drawn into the inner chamber.
5. Distillation is continued until the erlenmeyer is three-fourths full, at which time the flask is held below the condenser tube and the condensate is allowed to drip into the erlenmeyer for about 20 sec. The outside of the condenser tip previously in contact with the acid water is rinsed with ammonia-free water and also collected in the flask.

6. The solution in the erlenmeyer flask is then poured into a 50 ml volumetric flask. Two water rinses of the erlenmeyer are also poured into the volumetric flask and the solution is Nesslerized.

C. Cleaning of Distillation Apparatus

1. Empty inner chamber and rinse inner chamber at least three times with distilled H_2O.
2. Add 1 or 2 ml of 50% H_2SO_4 to addition funnel and fill addition funnel to top with distilled H_2O.
3. Fill the inner chamber with the acid water from the addition funnel. If the instrument is to be used immediately, the solution is then removed. If the instrument is to be stored, the acid water is left in the reaction chamber.

D. Nesslerization

1. Add 1 ml 10% NaOH (w/v) to steam distillate and washings in 50 ml volumetric flask and mix.
2. Add 5 ml Nesslers Reagent (Koch-McMeekin Formula, Scientif Products, Evanston, Ill.) to flask. Shake well.
3. Dilute to 50 ml, mix thoroughly and read OD on the Spectronic 20 at 480 mμ.

APPENDIX II (A)

PROCEDURE FOR AMMONIA ANALYSIS--

PERMUTIT METHOD

1. Add 670 mg dry Permutit-Folin (Arthur H. Thomas Company, Philadelphia, Pennsylvania) to each 50 ml volumetric flask.
2. Add 0.5 ml of 0.1 N H_2SO_4.
3. Pipette 25 ml of each sample into each of two volumetric flasks. Shake for 5 min.
4. Allow the Permutit to settle to the bottom and carefully decant off the supernatent, preventing loss of Permutit.
5. Add approximately 20 ml of acidified water to Permutit (2 ml concn. H_2SO_4 per liter distilled H_2O). Shake several times and decant supernatent. Repeat step (5).
6. Add 1 ml 10% (w/v) NaOH and shake.
7. Add approximately 30 ml distilled H_2O and Nesslerize as for total nitrogen (do not add 10% NaOH twice). See Appendix I.

APPENDIX II (B)

PROCEDURE FOR ANALYSIS OF UREA NITROGEN -- ENZYME DEGRADATION METHOD

1. To 50 ml volumetric flasks add 670 mg Permutit, 0.5 ml of 0.1 N H_2SO_4 and 25 ml of sample (duplicate determinations should be made).
2. Pipette 1 ml Urease enzyme (Urease Glycerol Extract, Scientific Products, Evanston, Ill.) into each flask and shake until thoroughly mixed.
3. Allow flasks to stand for 30 minutes at room temperature, shaking flasks occasionally during this period.
4. After 30 minutes shake flasks continuously for 5 minutes.
5. Follow steps 4 through 7, Procedure for Ammonia Determination -- Permutit Method.
6. The quantity of urea nitrogen is found by subtracting the quantity of nitrogen determined by the Permutit method from the quantity of nitrogen determined by the Enzyme Degradation method.

APPENDIX III

PREPARATION OF STANDARD CURVES

For total nitrogen, ammonia nitrogen, and urea nitrogen assays, standard curves were determined using nitrogen as dry $(NH_4)_2SO_4$. The protein standard used for the Lowery Protein Determination was Protein Standard, Human Crystallized Albumin (Dade Reagents, Miami, Florida).

Agreement of the ammonia, urea and total nitrogen procedures was tested by making a standard containing ammonia, urea, and an amino acid (alanine) in the following amounts: ammonia (ammonium sulfate) 150 μg/2 ml, urea 75 μg/2 ml, and alanine 75 μg/2 ml. Two ml of the standard solution was diluted to 25 ml aliquotes and the solutions were analyzed by the micro-Kjeldahl, Permutit and urease procedures. All three procedures agreed with $\pm$ 4 μg nitrogen.

Table 6. --Data for standard curves

Analytical test	Nitrogen concentration μg N/ml	% transmittance	O.D.
Total Nitrogen "Micro Kjeldahl"	0	100.00	0.000
	50	85.75	0.067
	100	70.25	0.153
	200	47.00	0.327
	250	39.50	0.403
	400	22.00	0.656
	500	14.75	0.830
Ammonia "Permutit"	0	100.00	0.000
	25	94.00	0.024
	25	92.75	0.033
	50	85.50	0.068
	100	71.50	0.146
	250	42.75	0.370
	250	42.25	0.376
Urea "Urease"	0	100.00	0.000
	25	94.00	0.027
	25	92.75	0.033
	50	84.75	0.072
	50	84.25	0.074
	100	72.50	0.139
	100	71.50	0.145
	150	59.50	0.226
	150	58.75	0.231
Protein "Lowery"	0	100.00	0.000
	25	85.00	0.071
	25	84.00	0.076
	50	74.75	0.126
	50	73.50	0.134
	100	55.25	0.258
	150	42.50	0.372
	200	33.00	0.482
	250	26.25	0.581

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