THE EFFECT OF AMBIENT AMMONIA LEVELS ON BLOOD AMMONIA AND AMMONIA EXCRETION OF RAINBOW TROUT (SALMO GAIRDNERI)

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
Janet R. Gillette
1967

ABSTRACT

THE EFFECT OF AMBIENT AMMONIA LEVELS ON BLOOD AMMONIA AND AMMONIA EXCRETION OF RAINBOW TROUT (*SALMO GAIRDNERI*)

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Blood ammonia concentrations of *Salmo gairdneri* were measured using the Conway technique and compared to the ambient ammonia concentrations to which these fish had been exposed for 24 hours. Nitrogen excretion rates for the fish were measured by comparing initial and final ambient concentrations of ammonia, measured by the permutit method, with initial and final total nitrogen concentrations, measured by the micro-Kjeldahl method. Fish exposed to 0, 1, 3, 5, and 8 μg/ml ammonia (NH_3 + NH_4^+) had blood levels of approximately 38, 42, 51, 59, and 71 μg/ml ammonia, respectively. Considering pH, these ambient solutions corresponded to approximately 0 to 1 μg/ml unionized ammonia (NH_3) with the corresponding blood range being ~~0.4~~ 0.6 to ~~1.4~~ 1.3 μg/ml unionized ammonia. In each case the blood ammonia levels were higher than the ambient ammonia levels. Total nitrogen excretion rates decreased from 250 μgN/g body wt./day for fish in 0 μg/ml ammonia to 180 μgN/g body wt./day for fish in 8 μg/ml ammonia; whereas the corresponding ammonia excretion rates

decreased from 130 µg/g body wt./day ammonia nitrogen to 50 µg/g body wt./day ammonia nitrogen. These data support the theory that ammonia is excreted via passive diffusion along a steep concentration gradient from blood to water across the gill surface. The decreased nitrogen excretion and increased blood ammonia concentrations of these fish suggest that the rate of this passive diffusion is retarded when the gradient is decreased; and accumulation of endogenous ammonia appears to result. The percent of total nitrogen excreted as ammonia decreased from 51.7% at 0 µg/ml of ambient ammonia to about 30% at 8 µg/ml of ambient ammonia, indicating increased excretion of another nitrogenous compound(s) in partial compensation for the reduced ammonia excretion.

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Physiology

1967

ACKNOWLEDGMENTS

The author wishes to express sincere gratitude to Dr. Paul O. Fromm for his guidance and encouragement which made this work possible.

Deep-felt appreciation is also expressed to Joe Abbate, Cliff Hill and the many other graduate students who helped so much throughout this study.

The author is indebted to Dr. J. R. Hoffert for his statistical advice.

The author is indebted also to the Federal Water Pollution Control Administration, Department of Health Education and Welfare for their financial support of this work. Funds were provided under grant number W P 00807.

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INTRODUCTION AND LITERATURE REVIEW

Nitrogen forms a part of important structural and metabolic components of all living organisms. There is a constant turnover of this element during many catabolic and anabolic processes. When substances such as proteins and nucleic acids are synthesized, nitrogen is incorporated, however, as these substances are broken down the nitrogen may be transferred to other compounds or eventually released in the form of ammonia. Unlike plants, animals have little capacity for the storage of ammonia nitrogen. Since there are large quantities of ammonia released during digestion of foods, in addition to the small amounts released during other metabolic activities, and since this substance is quite toxic, excess quantities of it must be eliminated from the animal. Animals have adapted to this situation by developing excretory mechanisms. When, however, a deficiency in these excretory mechanisms occurs, serious complications result.

Ammonia Nitrogen Metabolism

General Aspects

The actions of bacterial deaminases (Warren and Newton, 1959) and bacterial ureases (Fishbein et al., 1966)

on ingested foods contribute markedly to the production of ammonia in the gastrointestinal tract. This ammonia is then absorbed from the alkaline intestinal fluids into the portal circulation. Most of the absorbed ammonia (in the guinea pig) is then absorbed by the liver before entering the peripheral circulation (Warren and Newton, 1959).

The absorbed ammonia has three main fates:

1. Participation in metabolic processes whereby the following compounds are formed: amino acids, mucleotide bases, choline and acetylcholine, bases and phosphagens (creatine and creatine phosphate, arginine and arginine phosphate), and betaines.
2. Storage as glutamine.
3. Excretion as NH_3, urea, uric acid, or trimethylamine oxide.

The chart which follows lists important metabolic reactions for incorporation, transfer and release of ammonia-nitrogen.

The fixation of ammonia nitrogen occurs mainly via glutamate, glutamine and asparagine formation. The nitrogen may then be transferred to other useful metabolic compounds mostly via transamination reactions. In this way the ammonia nitrogen becomes a part of such important compounds as amino acids and nucleotides.

Ammonia is released by several deaminating enzymes and by some physiological processes, the exact nature of which cannot at present be explained biochemically. For example, nervous activity, according to Weil-Malherbe (1962),

AMMONIA NITROGEN INCORPORATION

	Site	Requirement
L-glutamic acid dehydrogenase: NH_3 + α-ketoglutarate yield glutamate (may occur in 2 steps)	hepatic mitochondria	NADH
Glutamine and asparagine synthesis:		
aspartate + NH_3 yield asparagine		ATP
glutamate + NH_3 yield glutamine		ATP
Synthesis of carbamyl phosphate: NH_3 + CO_2 + ATP yield carbamyl phosphate		ATP

NITROGEN TRANSFER REACTIONS

	Site	Requirement
Transamination: transfer of an amino group for a keto group	kidney heart liver	pyridoxal phosphate
Transimination transfer of amino group for a hydroxyl group	kidney liver	magnesium ATP
Transamidination: transfer of the aminine group of arginine to other compounds		

DEAMINATION REACTIONS

	Site	Requirement
Hydrolytic deamination: hydrolysis of amino acid with production of ammonia	intestinal bacteria	
Oxidative deamination: oxidation with molecular oxygen to yield the keto acid and ammonia	kidney, liver	
L-glutamic acid dehydrogenase: glutamate yields ammonia and α-keto glutarate		ADP NAD

is accompanied by a liberation of ammonia and the rate of its release has been correlated with the intensity of nervous activity. As early as 1922, Tashiro was able to demonstrate production of ammonia by an electrically stimulated excised frog nerve. Takashi, et al. (1961) found an increase in ammonia content of the brain as a result of convulsive activity. They suggest that this increase is a metabolic consequence of functional excitation of nerve tissue. In a recent review of the literature concerning ammonia metabolism in the brain, Weil-Malherbe (1962) reported that the ammonia level of the brain increases as a result of painful stimulation of the extremities, and during certain conditioned reflexes. These responses were noted in the absence of convulsions. Coversely, he described decreased brain ammonia levels produced during anesthesia and during sleep.

Weil-Malherbe postulates that oxidative phosphorylation is necessary for ammonia formation in the brain since evidence indicates the importance of intact mitochondria. In support of this view he presented data (Weil-Malherbe and Green, 1955) which showed that in the absence of oxygen or in the presence of 2:4 dinitrophenol ammonia formation in brain slices is reduced to the level found in homogenates where mitochondria are no longer intact. He further suggests that formation of ammonia in nervous tissue in vivo

and *in vitro* is due to reactions involving proteins and nucleoproteins.

Ammonia is produced during muscle contraction by some poorly understood processes. Schwartz *et al*. (1958) have demonstrated in humans the elevation of peripheral blood levels following convulsions due to Metrozol and after voluntary exercise. They suggest deamination within the muscle itself as the source of the released ammonia. Feinberg and Alma (1961) after studying ammonia production in isolated rabbit heart, suggest that the ammonia results from AMP deamination. Barnes *et al*. (1964) demonstrated a rise in blood ammonia in rats after muscular contraction and they showed that the rate of removal of the blood ammonia following exercise could be increased by continued daily exercise or daily feedings of ammonium carbonate. They suggest that both of these processes "cause adaptation of the enzymes of the ornithine cycle."

Excretory Forms of Waste Nitrogen

Waste nitrogen may be eliminated in several forms. A few invertebrates excrete excess nitrogen in the form of amino acids. Most aquatic invertebrates excrete their waste nitrogen as ammonia, while terrestrial invertebrates excrete mainly urea or uric acid. Other nitrogenous excretory products such as trimethylamine oxide, creatine, creatinine, purines and pyrimidines may also be excreted. Nearly all

animals excrete nitrogen in several of these forms, but usually one form predominates.

Baldwin (1952) postulates that the availability of water determines whether an animal will be ammonotelic or ureotelic. Aquatic vertebrates are usually ammonotelic. They rid the toxic ammonia via diffusion across the gills and by production of urine. The lung-fish is ammonotelic, but during the dry season it estivates as a ureotelic organism. Amphibia have adapted to the terrestrial environment by becoming ureotelic; by producing urea the animal can store waste nitrogen in a relatively non-toxic form until water is abundant enough that nitrogen can be eliminated via a highly concentrated urine. The frog is ammonotelic until metamorphosis and then becomes ureotelic. Some species retain the ability to revert back to ammonotelism when placed in water.

In those animals which do not have the advantage of an aquatic environment to dilute excreted ammonia, the production of ammonia may be deleterious if this substance is not eliminated by diffusion fast enough to prevent its accumulation within the animal. Uric acid and urea are less toxic than ammonia. Needham (1937) suggested that the nature of an animal's embryonic development determines whether an animal will be ureotelic or uricotelic. Those terrestrial animals which hatch from eggs maintained in a moist or wet environment produce urea which probably escapes into the

water of the immediate surroundings at a rate which is fast enough to prevent inhibition of ontogenesis. This is the case with some reptiles--they are ureotelic. The situation is quite similar with mammals for the embryo is bathed in fluid and nourished via the placenta which carries away the relatively non-toxic urea to be excreted via the maternal kidneys. The eggs of some animals e.g., birds and some reptiles, however, are supplied with only enough water to carry them through the embryonic development. These animals must deposit their nitrogenous wastes in the form of uric acid, an insoluble non-toxic substance that may accumulate within the egg without harm to the embryo.

Production of Ammonia by Excretory Organs

Kidney. Ammonia production in the kidney results mainly from breakdown of glutamate and glutamine (Van Slyke et al. 1943). Amino acids probably transfer their amino group to α-ketoglutarate, forming glutamate which then undergoes deamination, resulting in release of ammonia in the mammalian kidney (Richterich and Goldstein, 1958):

$$\text{amino acid} + \alpha\text{-ketoglutarate} \xleftrightarrow{\text{transaminase}} \text{glutamate} + \alpha\text{-keto acid}$$

$$\text{glutamate} + NAD^+ \xleftrightarrow{\text{glutamic dehydrogenase}} \alpha\text{-ketoglutarate} + NH_3 \text{ and } NADH + H^+$$

Pilkington (1965) demonstrated that excess ammonia and NADH shift the glutamic dehydrogenase reaction to the left.

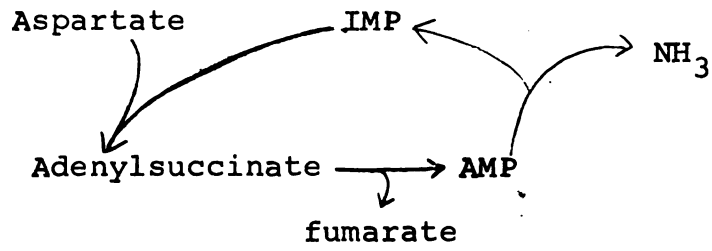
In dogs, the extraction of glutamine from the blood by the kidney has been observed by Van Slyke (1943).

$$H_2O + \text{glutamine} \xrightarrow{\text{glutaminase}} \text{glutamate} + NH_3$$

Ammonia is released from glutamine by the action of glutaminase in an essentially irreversible reaction. In the mammalian kidney glutaminase activity accounts for most of the ammonia produced (Makarewicz and Zydo, 1962). Goldstein (1966) has shown that in rats the glutaminase reaction is inhibited by glutamate, and Pilkington (1965) has observed inhibition of the reaction by ammonia as well. An additional source of renal ammonia may be AMP. Makarewicz and Zydowo (1962) observed activity of AMP-aminohydrolase and adenosine aminohydrolase in the kidneys of all animals tested (fish, frogs, chickens and mammals) except the tortise which has no adenosine aminohydrolase.

Gill. Both glutaminase and glutamic acid dehydrogenase activity have been observed in the fish gill by Goldstein and Forster (1961). Pequin and Serfaty (1966) found that glutamate dehydrogenase may not contribute to ammonia formation in carp (Cyprinus) gills. Makarewicz and Zydowo (1962) suggest that the activity of AMP-aminohydrolase is far more important than glutaminase for ammonia production

in the gill. They further suggest that the glutaminase becomes important for ammonia production only in higher animals.



Liver. Although some ammonia is produced by the gill (Goldstein and Forster, 1961), most of the ammonia excreted by ammonotelic animals such as the fish is formed by the liver (Pequin and Serfaty, 1963). McBean et al. (1966) have shown that in the liver the amino group of alanine is transaminated to α-ketoglutarate thus forming glutamate which is then acted upon by glutamic acid dehydrogenase to produce ammonia and α-ketoglutarate. Their suggestion that other amino acids follow the same reaction scheme for liberation of ammonia is in accord with the suggestion of Richerich and Goldstein (1958) concerning ammonia formation in the kidney.

McBean et al. (1966) further suggest that alanine and leucine are the only important "amine carriers" in fish because in Pequin and Serfaty's liver perfusion tests (1963) these two compounds were the only amino acids (other than glutamine) which gave rise to ammonia. They discount

glutamine as a "carrier" since Wu (1963) found that glutamine synthetase is present in fish only in brain tissue.

Excretion of Ammonia

Via the kidney. Although excretion of ammonia by the kidney allows elimination of waste nitrogen, the main function of ammonia in the kidney is its role in regulation of acid-base balance. It is generally considered that the movement of ammonia from the tubular cells to the urine is a passive process. Robinson and Owen (1965) have proposed that a diffusion equilibrium exists between the concentration of ammonia in the fluid of the loop of Henle, vasa recta blood and collecting duct fluid. Since only the non-ionic moiety is diffusable the diffusion of ammonia is pH dependent.

$$NH_3 + H^+ \longrightarrow NH_4^+$$

According to Pitts (1963), ammonia excretion depends on four factors in the mammalian kidney: (1) Urine pH, (2) NH_3 production rate, (3) Urine flow, and (4) Tubular cell pH. Sullivan and McVaugh (1963) postulate that blood pH is also important.

Production of ammonia is increased during acidosis (Pitts, 1963) and when the acidity of an animal's blood is increased much H^+ is excreted into the urine thus lowering the urine pH. Ammonia diffuses into the urine and is

trapped as the non-permeable ammonium ion. (Some blood ammonia is trapped in the same way by the acids of the stomach as shown by Fleshler and Gabuzda, 1965). More importantly, however, the H^+ is also trapped. As a result sodium may be reabsorbed in exchange for H^+.

Goldstein (1966) has suggested that the observed drop in glutamate concentration which accompanies acidosis may permit an increase in ammonia production by glutaminase activity. The cause of this decreased glutamate level is not clear, but Goldstein has raised the possibility that a direct link may exist between renal gluconeogenesis and ammonia excretion.

Via the Gill. Goldstein, et al. (1961, 1964) have shown that 60% of the ammonia excreted at the gill surface of a marine teleost (Myoxocephalus scorpius) results from a removal of blood ammonia by the gills. They suggest that although their findings may represent a carrier mediated transport, the simplest mechanism of excretion would be a non-ionic diffusion of unionized ammonia down a steep concentration gradient.

Maetz, et al. (1964) gives evidence to support the hypothesis that sodium is exchanged for ammonium across the gill surface of a fresh-water fish, Carassius auratus. They suggest that oxidative deamination by enzymes in the gill cells causes production of ammonia in molecular form (NH_3).

This ammonia is then converted to ammonium by addition of protons liberated from dissociation of carbonic acid. They propose an exchange of the ammonium ions for sodium ions and suggest, alternatively, that ammonia and the hydrogen ions originating from carbonic acid diffuse independently through the membrane in exchange for sodium ions with the protons then being captured by ammonia on the outside. They suggest, without conclusive data, that the influx of sodium ions decreases when external water ammonium is increased and that when the outside ammonium concentration exceeds the cellular ammonium concentration an efflux of sodium ions occurs.

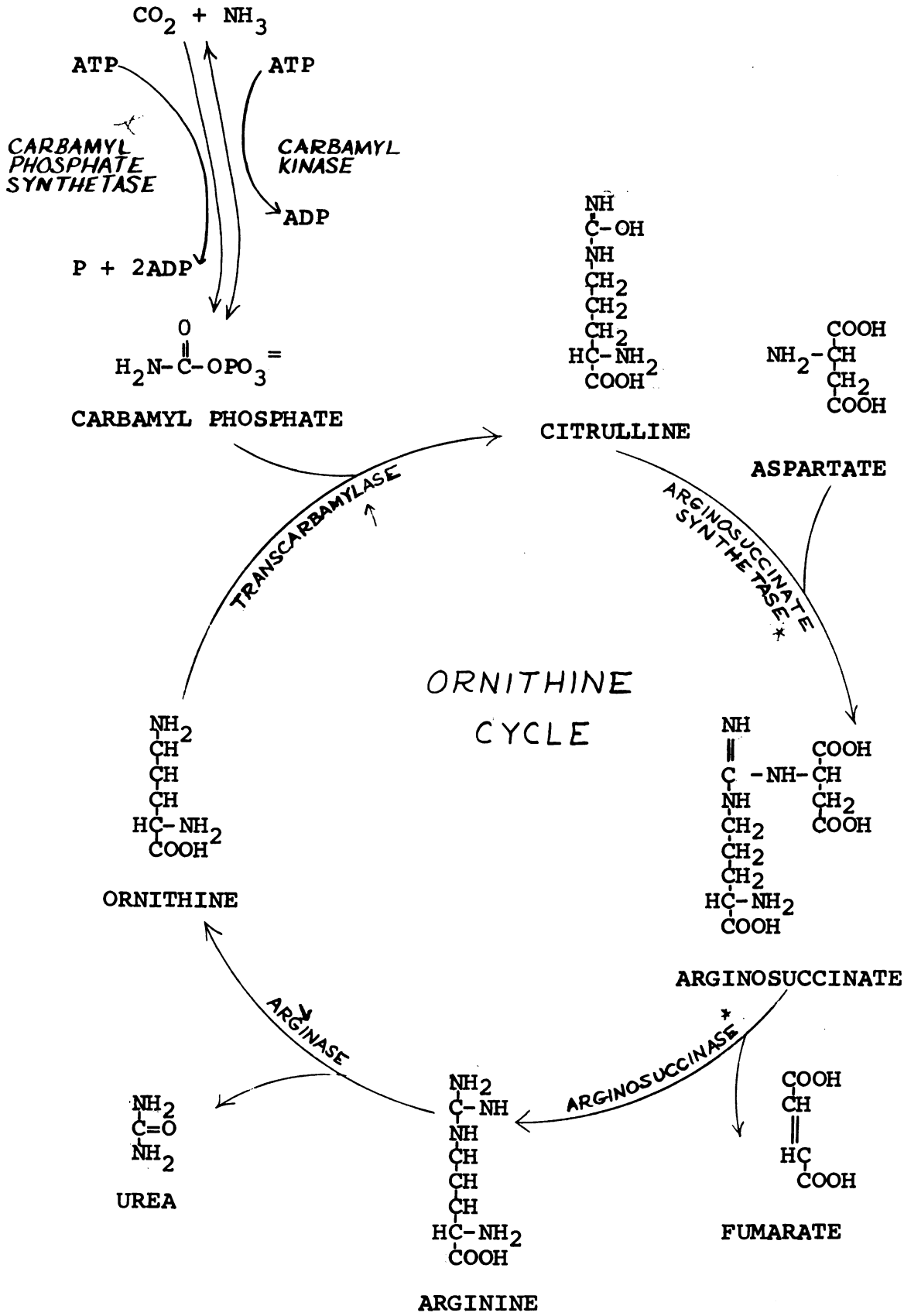
Of related interest are the experiments of Ryberg (1948) which support the idea that NH_4^+ and H^+ are excreted in the kidney by exchange with cations from the tubular fluid. He postulates an active exchange of NH_4^+ for sodium.

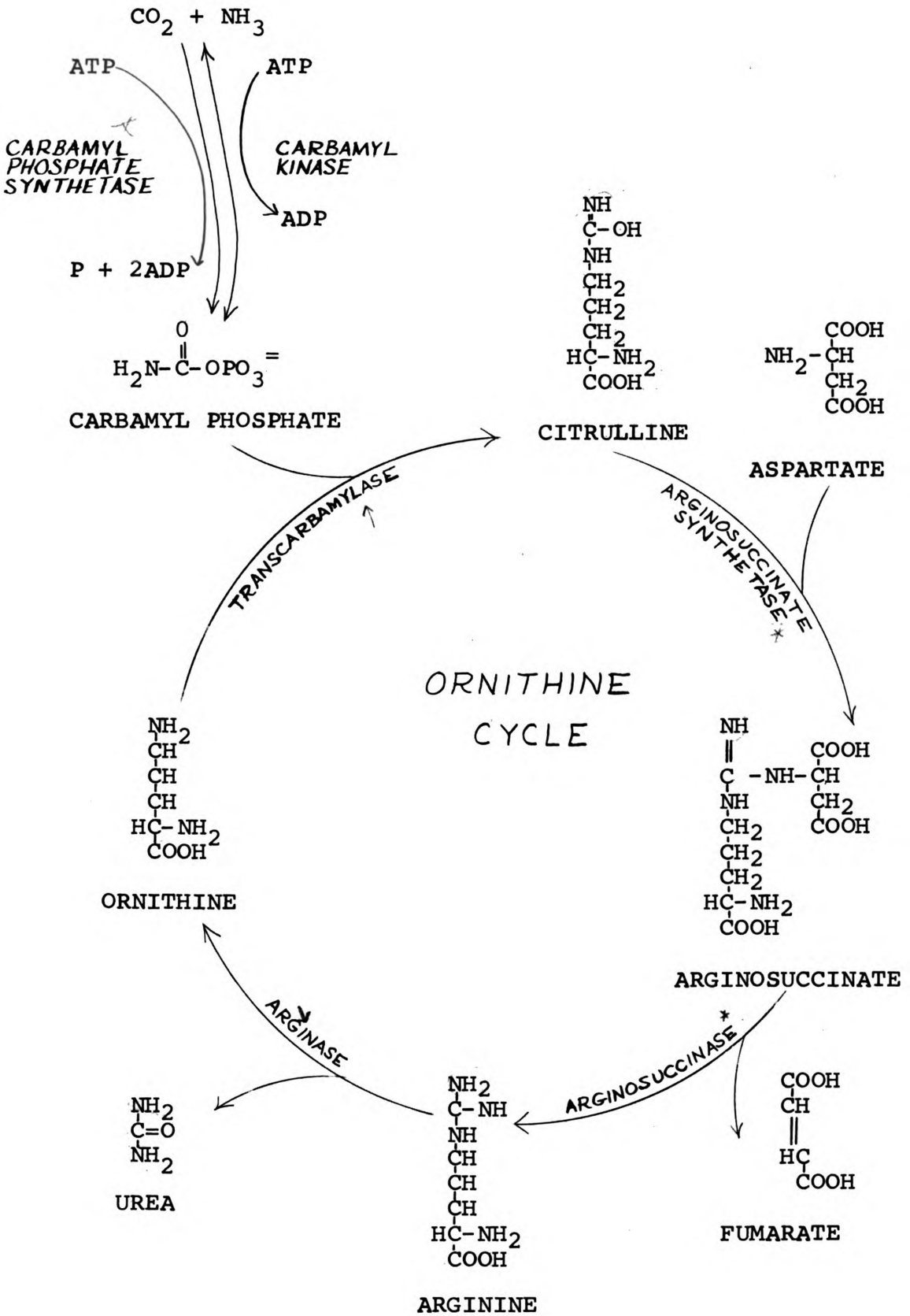
Production of Urea

The liver is the main site of urea synthesis (White et al., 1964) and in ureotelic animals contains all the enzymes of the ornithine cycle. This cycle requires 4 ATP per turn. A key enzyme is arginase which allows the irreversible formation of urea and ornithine from arginine. Since all cells contain the amino acid arginine, any cell which has arginase as well can produce urea. In 1959, Sporn et al. noticed urea synthesis in rat brain tissue. Later, Buniatian and Dautian (1966) observed pronounced arginase activity in

rat, frog, and chicken brain tissue. This tissue also contains argininosuccinate synthetase and arginosuccinase--enzymes necessary for synthesis of arginine from citrulline and aspartate, but the activity of the latter two enzymes in frog and chicken brain is very low, and the activity of arginase in the chicken brain decreases during embryonic development. The complete ornithine cycle in the brain tissue of these three animals is not present, however, because in each case the enzyme system synthesizing citrulline from CO_2, NH_3 and ornithine is absent.

The frog Rana catesbeiana represents an interesting intermediate between an ammonotelic animal like the fish and a ureotelic animal such as the rat. During metamorphosis this animal changes from an ammonotelic to a ureotelic organism. Brown et al. (1959) has observed a marked enhancement in the activities of all the liver enzymes of the urea cycle at the onset of metamorphosis in this species. They suggest induction of the urea cycle caused an increased de novo synthesis of the necessary enzymes. Prior to metamorphosis, the frog, like other ammonotelic animals, does not contain measurable or detectable quantities of carbamyl phosphate synthetase (Brown and Cohen, 1960). During metamorphosis, however, the level of this enzyme increases and shows activity (Cohen and Sallach, 1961, Florkin and Mason, 1964). White et al. (1964) suggests that non-ureotelic animals contain carbamyl kinase which allows a reversible synthesis of


$CO_2 + NH_3$
ATP
ATP
CARBAMYL PHOSPHATE SYNTHETASE
CARBAMYL KINASE
ADP
P + 2ADP
CARBAMYL PHOSPHATE
CITRULLINE
ASPARTATE
ARGINOSUCCINATE SYNTHETASE
ARGINOSUCCINATE
ARGINOSUCCINASE
FUMARATE
ARGINASE
ARGININE
UREA
ORNITHINE
TRANSCARBAMYLASE
ORNITHINE CYCLE


$CO_2 + NH_3$
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ASPARTATE
TRANSCARBAMYLASE
ARGINOSUCCINATE SYNTHETASE
ORNITHINE CYCLE
ORNITHINE
ARGINOSUCCINATE
ARGINASE
ARGINOSUCCINASE
UREA
FUMARATE
ARGININE

carbamyl phosphate thus permitting return of the N to the NH_3 pool.

Increased de novo synthesis has also been found for the enzymes arginosuccinate synthetase and arginosuccinase. These enzymes catalyze rate-limiting steps of the ornithine cycle.

Toxicity of Ammonia

Toxicity of Ammonia to the Respiratory Surface

Toxic effects of ammonia are related both to concentration and length of exposure. When 6 men were exposed to 50 ppm ammonia gas for several hours, they suffered considerable discomfort manifest by excessive nasal and lacrimal secretion and coughing (Anderson et al., 1964). High concentrations of ammonia may lead to intense congestion and swelling of the upper respiratory passages. Death may result from spasm or edema of the larynx (Henderson et al., 1943).

When chickens were exposed to 200 ppm ammonia gas they exhibited edema, congestion, dilation of veins and some hemorrhage (Anderson et al., 1964). When chickens were exposed to 20 ppm ammonia these workers could not find visible effects due to ammonia until after six weeks of exposure. Deleterious effects due to ammonia were indicated, however, when chickens exposed to 20 ppm ammonia for 72 hours were

also exposed to Newcastle disease virus. The infection rate for this disease was increased.

Burrows (1964) found similar responses in fish exposed to sublethal levels of dissolved ambient ammonia in their water. These fish exhibited a decrease in stamina and an increased incidence of bacterial gill disease. Histological examination revealed proliferation and consolidation of the gill lamellae. An increase in mucus secretion was also noted.

The above effects may result from external irritation of ammonia at the respiratory surface or from internal effects due to a rise in ammonia concentration within the animal. Ammonia is soluble in water and could possibly dissolve in the moist respiratory surface. If this happens it is possible that the ammonia could diffuse inward and be distributed within the animal via the blood stream.

Toxicity of Ammonia to Fish

Ammonia, the main nitrogenous excretory product of fish (Denis, 1913-14; Smith, 1929 and Fromm, 1963), and one of the more common pollutants discharged into streams (Klein, 1959 and Lloyd, 1961) is very toxic to fish. Numerous observations have been made concerning the toxicity of ammonia, but the mechanism of its action is not yet understood.

General aspects of ammonia toxicity. It is the unionized form (NH_3) ambient ammonia which appears to be

toxic. Thus, the concentration of toxic ammonia is an aqueous medium depends on both the pH (Ellis, 1937; Grindley, 1946; Wuhrmann, 1948 and Lloyd, 1961b) and temperature (Wuhrmann, 1953). Hydrogen ion concentrations between pH 5.0 and 9.0 are not lethal to fish (Doudoroff and Katz, 1950).

The severity of ammonia toxicity is also increased by raising the temperature of an ammonia solution, and in turn that of the fish, thereby causing increased metabolic rate. Increased metabolism causes increased respiratory flow which, according to Lloyd and Herbert (1960), may increase the rate at which the toxic ammonia is absorbed. At the same time a rise in temperature stimulates a greater production of ammonia by the animal. This temperature effect must be considered when fish are to be safely transported in sealed containers (Summerfelt, 1967; McFarland, 1960 and Gebhards, 1965). Temperature has a direct effect on an ammonia solution by decreasing the pKa value and thus, influencing the concentration of unionized ammonia.

Carbon dioxide and oxygen content may significantly alter the toxicity of a given concentration of unionized ammonia. Downing and Merkens (1955, 1957), Downing (1957), Lloyd (1961a, 1961b), Burrows (1964) and Gebhards (1965) all have shown that decreased oxygen availability greatly enhances ammonia toxicity. Moreover, carbon dioxide accumulation will increase the toxicity (Alabaster, 1957; Downing

and Merkens, 1957; Lloyd and Herbert, 1960 and Gebhards, 1965), but its effect can be counteracted by raising the levels of oxygen in the water (Gebhards, 1955).

Warren and Shenker (1962) have found that pH changes caused by CO_2 do not produce the same effects as do pH changes resulting from addition of a fixed acid or base. They attribute this difference to the ability of CO_2 to freely diffuse across the membrane of mammalian cells. This diffusable CO_2 lowers the pH on both sides of the cell membrane. Warren and Schenker also assume that only the unionized ammonia is freely diffusable. Thus, when the cells are bathed by an ammonia solution the concentration gradient of the unionized ammonia determines what movement of ammonia will occur. Since, reduction of pH in the bathing solution by addition of CO_2 may be concurrent with a similar reduction of pH within the cells, the concentration of unionized ammonia is decreased on both sides. When fixed acid is introduced into the bathing media, however, the concentration of ammonia is decreased only on the outside. An efflux of ammonia results. These workers suggest that similar processes effect the transfer of ammonia across the gill surface when CO_2 is added to aquarium water containing ammonia.

If unionized ammonia effects the oxygen transport system of the blood as suggested by Brockway (1950), increased carbon dioxide and/or decreased oxygen could certainly amplify the toxic effects of this substance as would

temperature increases. Wuhrmann (1948) and Gebhards (1965) infer that ammonia increases fragility of erythrocytes. These suggestions are in accord with the hypothesis of Brockway.

Other workers have observed an effect on the nervous system due to the increased levels of ammonia. Wuhrmann (1948) reports that the first noticeable effect of ammonia poisoning is on the nervous system causing "ammonia cramps" and then convulsions. These observations are in accord with a previous report by McCay and Vars (1930) who relate that fish exposed to toxic levels of ammonia suddenly swim madly about the aquarium and may even leap from the water.

Experimentation

If, as suggested, the unionized ammonia affects both the oxygen carrying capacity of the blood and the nervous system, it appears probable that blood levels of unionized ammonia are increased when fish are placed in ammonia solutions. This rise in blood ammonia could be accounted for by an inhibition of the excretory process whereby ammonia is diffused outward at the gill, and/or by an inward diffusion of ammonia. Accumulation of ammonia within the fish may be of greater importance than the external effects of ammonia mentioned above.

The purpose of the work described here was to demonstrate what changes occur in blood ammonia levels and in

ammonia (or ammonium) excretory rate when fish are exposed to increased concentrations of ammonia. Both total ammonia (NH_3 and NH_4^+) and unionized ammonia levels in the blood and in ambient water solutions are compared to show whether or not blood levels increase in the fish exposed to the high ammonia concentrations. Data for 24 hour excretion of total nitrogen and ammonia nitrogen were obtained in order to determine if any change in rate or form of nitrogenous waste excretion occurred in fish exposed to various concentrations of ambient ammonia.

METHODS AND MATERIALS

Experimental Animals

The animals used were rainbow trout (Salmo gaidneri), obtained from the Michigan Conservation Department in Grayling, Michigan. They were transported to Michigan State University in a large galvanized metal tank which was coated inside with non-toxic paint. The water was agitated to provide continual aeration.

The fish were kept in rooms maintained at 12-13°C. The lights were held on a daily cycle which provided light from 7 AM to 9 PM.

The fish were stored in 300 liter fiberglass covered wooden tanks. Tap water was charcoal filtered to remove chlorine and excess iron, and run into the tanks via a flow-through system which allowed continuous exchange of the water. Aeration was provided via air lines held at the bottom of the tank. Small charcoal filters were inserted into each air line to trap oil.

Twice a week the fish were fed commercial trout pellets. Before a group of fish was used in an experiment the fish were transferred to small (100 liter) tanks held under the same water, light and temperature conditions.

These fish were starved for 7 to 10 days to allow a leveling off of the precipitous drop in both ammonia nitrogen and total nitrogen excretion which occurs during the first 6 days of starvation (Fromm, 1963). Thus, the nutritional status of the fish was controlled and at the same time fecal deposition which might interfere with the experiment was reduced.

Experimental Design

Starved fish were placed in small plastic tanks containing 3.5 liters of aerated tap water containing the following concentrations of ammonia:

o µg/ml
1 µg/ml
3 µg/ml or 5 µg/ml
8 µg/ml

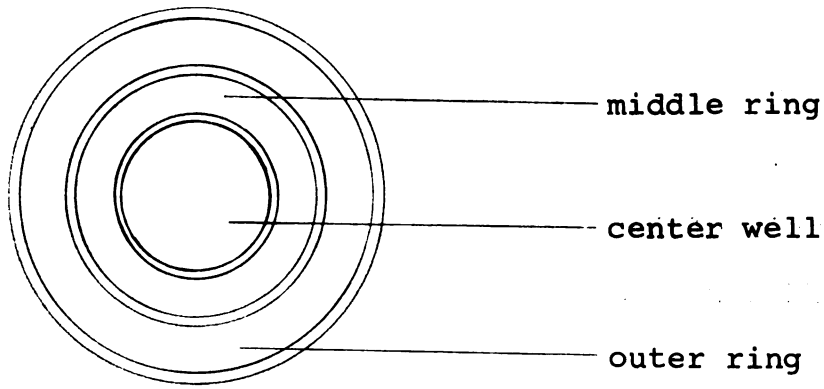
Eight fish were used at a time with 2 fish at each concentration. The fish tanks were covered, and a curtain was drawn in front of the fish to prevent excitation of the fish when people entered the room. This room was maintained with the same temperature and light conditions prevailing as were in the fish storage rooms.

After 24 hours the fish were anesthesized with tricainmethane sulfonate (MS 222; Sandoz Pharmaceuticals, Hanover, N.J.). Blood samples were taken by syringe via puncture of the dorsal aortic arch and were placed immediately

into Conway diffusion dishes for analysis of blood amonia nitrogen. This analysis was carried out in the same room at 10-12°C. Water samples were collected for ammonia nitrogen, total nitrogen and pH determinations. Since nitrogen excretion varies with body weight, the weight of each fish was recorded.

Blood Ammonia--Conway Method

The method for determining blood ammonia was based on the work of Conway (1933, 1939 and 1963) and of Tashiro (1922). The modified Conway units were designed by Obrink (1955). These units were made of polypropylene, a chemically inert plastic.



Conway Unit

Reagents:

Tashiro's Reagent. Two hundred ml. of a 0.1% alcoholic solution were prepared. To this were added 50 ml. of

a 0.1% alcoholic solution of methylene blue. The solution was then transferred into a brown bottle where it may be kept indefinitely.

Standard HCl with indicator. Five ml. of Tashiro's reagent were run into a 500 ml. flask. One hundred ml. of absolute alcohol were added. The flask was filled up to about 3/4 the volume with ammonia free distilled water containing 0.005% Tergitol nonionic NPX detergent (Union Carbide Chemicals Co.). The indicator was brought to a neutral point by dropping in a little dilute alkali until the red color was gone. To this were added 33.3 ml. of 0.1N stock solution of HCL. More ammonia free water was then added until the final solution was brought up to the 500 ml. mark. (Ammonia free water was prepared by adding permutit to the distilled water carboys and then siphoning water off the top.)

Stock barium hydroxide. A 0.1 N barium hydroxide solution was prepared by dissolving 1.5775 g of $Ba(OH)_2$ (formula weight of $Ba(OH)_2 \cdot 8H_2O$ = 315.5) in 100 ml. of ammonia free water. Precipitates (barium carbonate) which formed were immediately filtered out.

0.014 N. barium hydroxide. Into a 250 ml. volumetric flask were poured 35.7 ml of the 0.1 N barium hydroxide stock solution. Ammonia free water was then added to the mark. This solution was poured into a burette reservior, and a sodalime trap was placed over the top opening to prevent CO_2

from entering. The solution was then titrated against a 0.001 N HCl solution containing phenolphthalein. If the barium hydroxide solution was not exactly 0.014 N. it was discarded. A fresh solution was prepared approximately every two days.

50% KOH. A 50% KOH solution was prepared by dissolving 50 grams of KOH in water and bringing the volume up to 100 ml.

Procedure. The Conway units were prepared by adding 1 ml. of a 0.0067 N HCl solution containing Tashiro's indicator into the center well via a microburette. One ml. of 50% KOH was added to both the middle ring and the outer ring. The cover was placed ajar on the unit so that it did not rest in the outer ring of KOH. One half ml. of blood sample was then placed across from the KOH in the middle ring immediately after withdrawal of the sample from the fish. The cover was placed in position and twisted until a seal of KOH was obtained. The unit was then rotated carefully to allow mixing of the KOH and blood sample.

The strong base allows conversion of all the blood ammonia to the NH_3 form. This NH_3 is volatile and diffuses into the center well of acid which traps the NH_3 as NH_4^+. The NH_3 cannot escape from the unit because of the KOH seal.

After exactly twenty minutes the unit was opened and placed on a magnetic stirrer. A tiny magnet was placed in

the center well. Barium hydroxide was titrated via a microburette into the pink acid until a green color was visible. The amount of barium hydroxide used was recorded.

Before each series of blood samples were run the barium hydroxide solution was titrated against a 0.001 N. HCl solution containing phenolphthalein to check the normality of the base.

The ammonia that is trapped in the center well binds free H+ thereby increasing the pH. Therefore, the acid from the units containing the higher concentrations of ammonia, required less barium hydroxide to reach the end point (green color). The volume of barium hydroxide used to titrate the blank (0 µg/ml) was subtracted from the volume used for each of the standards and each of the samples. A standard curve was obtained using NH_4Cl standards of 0, 20, 40, and 60 µg NH_4^+/ml.

Cleaning. The conway units were washed several times with tap water and soaked for a minimum of 30 minutes in a 0.001 N HCl solution with Tashiro's indicator. Providing the indicator did not change from pink to green, the dishes were then rinsed twice with distilled water and then two more times with ammonia-free distilled water. It was imperative that the dishes be clean in a "pH sense."

Water Ammonia--Permutit Method

Water ammonia was measured using the permutit method as described in Oser (1965) with sample sizes of 40 or 80 ml. depending on the approximate concentration to be measured. Ten ml. of Nessler's reagent were added and the samples to be read were brought up to 50 ml. with ammonia free water before being measured on a Coleman spectrophotometer at 480 or 410 mμ.

Note: Initially 480 mμ was the wavelength used as suggested by Oser (1965). Later, however an absorption spectrum was run on a sample using the Beckman Model D B-G grating recording spectrophotometer. This instrument revealed a definite peak absorbance at 400 mμ. Since the Coleman spectrophotometer the blank could not be set at 100% transmission at a wavelength of 400 mμ, a wavelength of 410 was used instead.

A standard curve was established for both of the wavelengths used. The standards ranged from 0 to 10 μg/ml NH_4^+.

Total Nitrogen--Folin Farmer Micro-Kjeldahl Method

Water samples were digested with concentrated sulfuric acid and hydrogen peroxide. Then, after steam distillation of these samples the total nitrogen was measured by direct Nesslerization. The procedure is outlined in Oser

(1965). A standard curve was prepared for 0 to 20 µg/ml. nitrogen.

Ammonia nitrogen produced as a result of bacterial deamination of any excreta present in the water samples has been found to be insignicant (Brown, 1958) and therefore, was not considered during these experiments.

pH Measurements

A Beckman Model G pH meter was used for all pH measurements. The pH of the blood was assumed to be 7.6 as found by Hoffert and Fromm (1966).

RESULTS AND DISCUSSION

Results

Blood Ammonia

When rainbow trout were subjected to increasing concentrations of unionized ammonia the levels of unionized ammonia in the blood increased. This relationship is linear. In each case the concentration of blood ammonia was higher than ammonia levels in the water from which the fish were taken. (Figure I).

Although pH variations in the water samples collected resulted in differing proportions of NH_3 and NH_4^+, the NH_3 concentration in a sample of higher total ammonia was nearly always greater than the NH_3 concentration in a sample of lower total ammonia. For this reason the total ammonia (NH_3 and NH_4^+) values for blood and water demonstrate the same relationships as for the unionized ammonia (Figure 2).

Nitrogen Excretion

As the water ammonia levels increased the total nitrogen excretion rates decreased (Figure 3). The ammonia excretion values decreased also but at a faster rate indicating that the reduction in ammonia excretion is to some

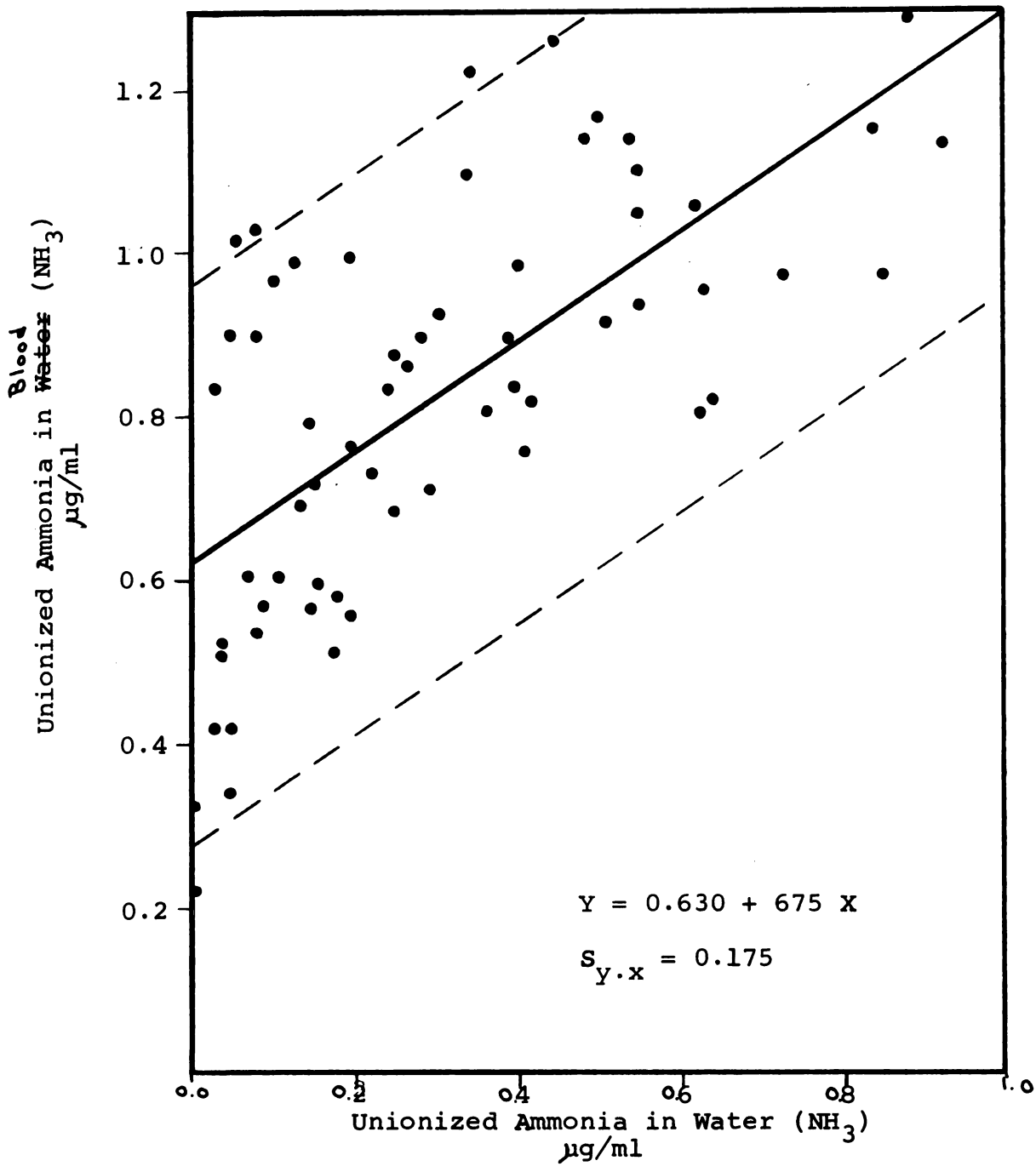


Figure 1. Unionized ammonia concentration in the blood as a function of unionized ammonia concentration in the water. The solid line was drawn by the method of least squares. Ninety five percent confidence limits for any point on this line are indicated by the dotted lines. The unionized ammonia values of the water were obtained by taking an average of the initial unionized ammonia level and the final level (after twenty four hours).

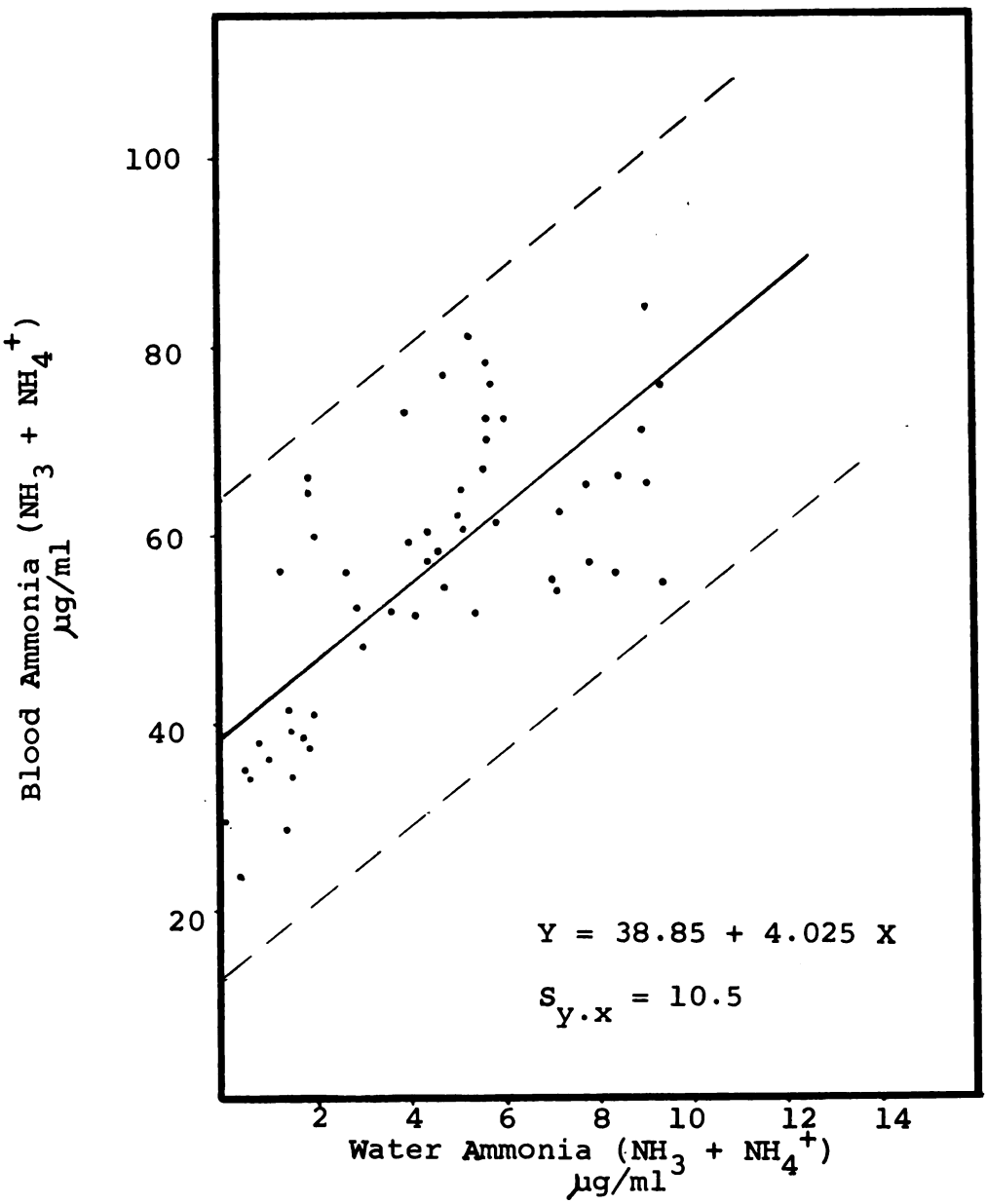


Figure 2. Ammonia concentration in the blood as a function of ammonia concentration in the water. The solid line was drawn by the method of least-squares. Ninety-five percent confidence limits for any point on this line are indicated by the dotted lines. The ammonia values of the water were obtained by taking an average of the initial ammonia level and the final level (after twenty four hours).

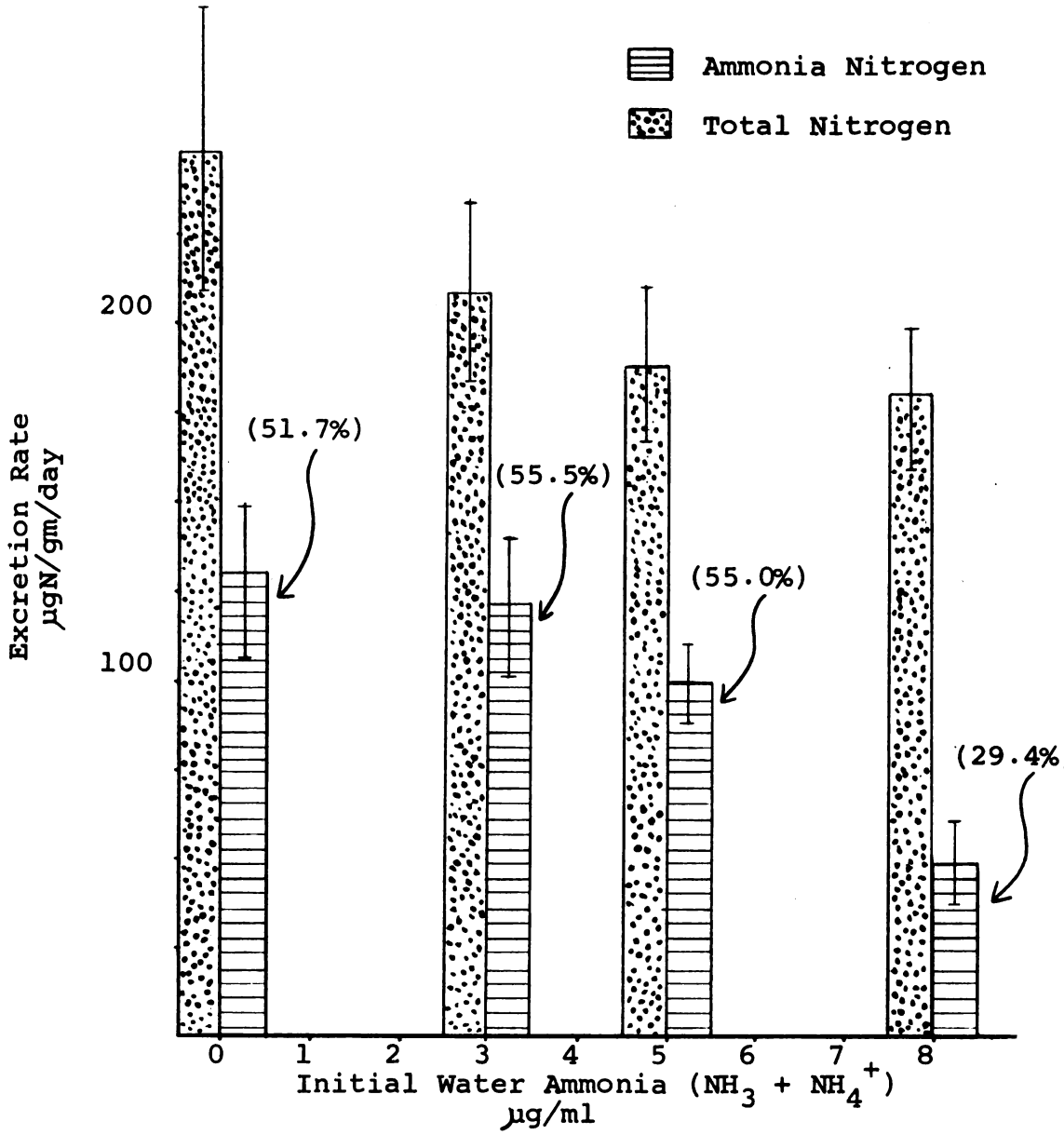


Figure 3. Total nitrogen and ammonia nitrogen excretion rates as a function of initial water ammonia. The percent of total nitrogen excreted is ammonia (NH_3 + NH_4^+) is given in parenthesis.

extent being compensated for by an increased excretion of some other nitrogenous compound.

Units for expression of nitrogen excretion. Nitrogen excretion values were obtained by measuring water nitrogen in units of μgNH_4^+/ml. The initial values were subtracted from the final values after 24 hours had elapsed to give μgNH_4^+/ml/day. This value was then multiplied by the volume of water in the tank and divided by the weight of the fish, give units of $ugNH_4^+$/g body weight/day. To convert into units of ugN/g body weight/day, all values were multiplied by 0.78. (NH_4^+ molecular weight = 18, N molecular weight = 14; $\frac{14}{17} = .78$.)

Discussion

Blood Ammonia

When the water ammonia concentration was increased, a rise in the blood levels of ammonia was observed and yet the blood levels of ammonia were consistently higher than the water levels of ammonia. These findings indicate that unionized ammonia may be excreted via a passive diffusion along a steep concentration gradient from blood to water across the gill surface as postulated by Goldstein (1964). Reduction of this gradient by increasing ambient ammonia concentration, which led to a decrease in rate of ammonia excretion also supports the idea that ammonia is excreted via a passive

diffusion. Ammonia is apparently produced at a faster rate than it can be eliminated under these conditions and blood levels of ammonia rise. The factors controlling gill excretion of ammonia seem to parallel those factors which Pitts (1963) suggests control ammonia excretion in the kidney.

Since some of the blood ammonia results from deamination of glutamate in a reaction which yields hydrogen ions in addition to NH_3, the hydrogen ions produced must be handled by the organism to prevent significant alteration of pH. Removal of the hydrogen ions might be accomplished by the action of buffers, by utilization of the ion in synthetic reactions or by excretion of the ion--perhaps by the mechanisms which Maetz (1964) described for the gill.

Toxic responses due to increased blood levels of ammonia in fish have not yet been investigated; however, this aspect of ammonia toxicity has been widely studied in several mammals. Rosado, et al. (1962) found that when blood levels of ammonia are raised by injection of NH_4Cl into the blood stream of dogs, much of the ammonia is rapidly removed by muscle (75%), liver (2.5%) and brain (0.5%) tissues. They suggest that the uptake of ammonia by muscle is an active process involving the exchange of NH_4^+ for K^+ ions. Glutamine synthesis was noted in the brain and liver cells, but muscle did not appear to be capable of synthesizing this compound. The trapped or bound ammonia is then released by

muscle cells and brain cells at a rate which is compatible with ornithine cycle activity for urea synthesis in the liver.

The ammonia concentration of brain tissue depends on the metabolic processes in the brain which produce ammonia, the regulatory nature of the blood brain barrier through which blood ammonia must pass if it is to enter brain tissues, and the detoxification mechanisms of the brain which bind ammonia into non-toxic compounds (Navazio, et al., 1961). Distribution of ammonia across the blood-cerebrospinal fluid barrier in humans follows the nonionic diffusion theory (Moore, et al., 1963). According to Navazio, et al. (1961) the pH of rat brain tissue is lower than that of rat blood, and therefore the concentration of ammonia (NH_3 and NH_4^+) in the brain tends to be higher than in the blood because NH_3 is trapped as NH_4^+ at the low pH.

Some of the ammonia detoxification mechanisms which have been observed in mammalian brain tissues are summarized by Weil-Malherbe (1962). The main "binding mechanism" is a combination of α-ketoglutarate with one molecule of ammonia to form glutamate and then with another molecule of ammonia in an endergonic process to form glutamine, a compound which more easily traverses the blood brain barrier than does glutamate. This compound may represent an electrically neutral transport form of ammonia. Ammonia may also give rise to aspartate and alanine as a result of transamination

reactions involving oxaloacetate and pyruvate. These detoxification reactions may lead to decreased ATP levels in the brain and interference with the citric acid cycle. Under these conditions respiration and glycolysis are stimulated, but with decreased function of the Krebs' cycle, accumulation of pyruvate and lactate results. According to Weil-Malherbe the toxicity of ammonia is so severe that "intracellular accumulation of ammonia must be prevented even though a high price may have to be paid for it" and in many cases the toxic effects associated with increased blood levels of ammonia are in fact due to changes brought about by functioning of the detoxification mechanisms.

McKhann and Tower (1961), however, propose that a primary toxic effect of ammonia in the brain may be direct interference with the oxidative decarboxylation of pyruvic acid and α-ketoglutarate. These two reactions are quite similar and involve the same cofactors, such as ATP and thiamine pyrophosphate. These workers relate an interesting communication with Dr. K. S. Warren in which an increased potency of ammonia toxicity in thiamine deficient rats is described. The glutamic dehydrogenase and glutamine synthetase reactions are protective mechanisms, according to McKhann and Tower (1961), which help maintain low levels of free ammonia and preserve oxidative metabolism. Moreover they suggest that an alternative pathway between the α-ketoglutarate to succinate stage (present only in the central

nervous system gray areas) of the citric acid cycle may independently support oxidative metabolism. This would provide a "safety-valve" mechanism for maintenance of metabolism around any block of α-ketoglutarate oxidative decarboxylation.

Schenker and Mendelson (1964) report normal ATP levels in rats with ammonia induced coma, suggesting no general defect in cerebral ATP synthesis nor widespread utilization of this compound. ATP may, however, be replenished by substances such as creatine phosphate, but these compounds were not measured. McKhann and Tower (1961) found no inhibition of the electron transport chain due to ammonia. As mentioned by Schenker and Mendelson (1964), there might be some compartmentalization for ammonia metabolism, and thus changes in ATP concentration within localized areas may occur.

When ammonia is not detoxified within mammalian systems, physiological changes may result. These changes are manifest by increased respiration (Schwartz, et al., 1958 and Rosado, et al., 1962), spastic muscular contractions (Greenstein, 1956), occasional tetanus (Navazio, et al., 1961), and coma (Navazio, et al., 1961). Warren and Schenker (1960) state that there may be a synergistic effect of hypoxia on ammonia toxicity. They correlate with this the observation that the neurological syndrome that most closely resembles

coma, which results from increased ammonia due to liver disease, is chronic pulmonary insufficiency.

Although it is well established that ammonia affects the nervous system of mammals little is known concerning the effect of ammonia on the nervous systems of lower animals. However, *in vitro* a depolarizing effect of ammonium ions on frog neurons (Lorente de No', *et al*., 1967) and squid giant axons (Tasaki, *et al*., 1965, 1966) has been noted.

Perhaps in fish also, the toxic effect of increased blood ammonia levels is manifest by changes in the nervous system. In the present work and in previous work (Wuhrmann, 1948 and McCay and Vars, 1930) fish placed in high concentrations of ammonia were seen to be hyperexcitable.

Other than to show that blood levels of ammonia rise, the present study on fish does not provide evidence to support or to disclaim the hypothesis of Brockway (1950) that ammonia affects the oxygen carrying capacity of the blood. The observed enhancing effects of increased carbon dioxide (Alabaster, 1957; Downing and Merkens, 1957; Lloyd and Herbert, 1960 and Gebhards, 1965) and/or decreased oxygen (Downing and Merkens, 1955, 1957; Downing 1957; Lloyd, 1961a, 1961b; Burrows, 1964; and Gebhards, 1965) on the toxicity of ammonia may be due to the direct effect of ammonia on oxygen carrying mechanisms as Brockway (1950) suggests, or to a synergistic effect which varies concentrations of carbon dioxide, oxygen and ammonia exert on the nervous system.

Nitrogen Excretion

Data presented lend support to the idea that ammonia is excreted via passive diffusion along a concentration gradient across the gill from blood to water. When fish are placed in ammonia solutions the gradient is reduced and ammonia cannot be eliminated fast enough to remove excess ammonia produced by the animal. This hypothesis, based on the occurrence of high blood ammonia values, is supported by the nitrogen excretion data which showed decreased nitrogen excretion rates in fish which were exposed to ammonia solutions.

In addition, the data indicate increased excretion of some other nitrogenous compound in partial compensation for the reduced excretion of ammonia. This substance is probably urea or amino acids. The production of urea in fish has been well established, however, all experimental evidence for the occurrence of the ornithine cycle in teleosts is negative (Brown and Cohen, 1960). The levels of carbamyl phosphate synthetase and ornithine transcarbamylase have been below the level of detection in all teleosts tested, including Salmo trutta, the brown trout. During metamorphosis of a tadpole, enzyme induction results in ornithine cycle activity and subsequently increased production of urea. It is conceivable that fish with impaired excretion of ammonia could somehow induce production of the ornithine cycle enzymes. A simpler way to increase urea

production in the rainbow trout, however, would probably be to increase arginase activity and production of arginine. Thus, without the complete ornithine cycle which feeds free ammonia into urea production, ammonia could first be incorporated into glutamate and then via transamination become part of arginine which is subsequently cleaved by arginase to yield urea and ornithine.

Before these hypotheses are tested, preliminary experiments should be conducted to show that increased production and excretion of urea does occur and not merely increased excretion of amino acids. Since increased production of amino acids is one of the detoxification mechanisms for ammonia found in mammals, it is quite possible that in fish with high blood ammonia values, production and excretion of amino acids is increased.

SUMMARY AND CONCLUSIONS

1. Rainbow trout were placed in solutions of 0, 1, 3, 5, and 8 µg/ml of ammonia; the respective blood ammonia concentrations for these fish were approximately 38, 42, 51, 59, and 71 µg ammonia/ml. Thus, there is a correlation between ambient ammonia and blood ammonia concentrations. (These same data may be expressed in terms of unionized ammonia. Fish exposed to a level of unionized ammonia between 0 and 1 µg/ml had blood ammonia values between ~~0~~ 0.6 and ~~1~~ 1.3 µg/ml.)
2. In each case the blood level of ammonia was higher than the water level of ammonia.
3. Ammonia seems to be excreted via passive diffusion across the gill surface.
4. The rate of this diffusion appears to depend on the gradient between blood ammonia and water ammonia. When this gradient was reduced, excretion of ammonia was retarded. Ammonia excretion dropped from 130 µg/g body wt./day for fish in 0 µg/ml ammonia to 50 µg/g body wt./day for fish in 8 µg/ml ammonia.
5. The increased blood ammonia is probably due to accumulation of endogenous ammonia rather than to a pronounced

influx of ambient ammonia, although measurements of the latter were not made.

6. The percent of total nitrogen excreted as ammonia decreased from 51.7% for fish exposed to 0 μg/ml ammonia to about 30% for fish exposed to 8 μg/ml ammonia. This suggests that increased excretion of other nitrogenous compounds occurred when ammonia excretion was inhibited by reduction of the ammonia gradient.

APPENDIX

Comment on the Blood Ammonia Analysis

Although contamination of the whole blood samples may have resulted from deamination of blood proteins, TCA (trichloroacetic acid) was not used to precipitate blood proteins as suggested by Nathan and Rodkey (who used a slightly different assay technique) because this compound interfered with the Conway assay for blood ammonia.

With TCA. When TCA was used the ½ ml blood samples were added to 1 ml. of chilled 20% TCA and centrifuged for ten minutes. One half ml. of the supernatent was then introduced to the Conway unit for ammonia analysis. The TCA caused an increased diffusion time, apparently, and 4 hours had to be allotted before reproducible results could be obtained with standards. This meant that at least 4½ hours passed before the sample could be analyzed.

Without TCA. Better results were obtained by placing the whole blood sample directly into the Conway unit immediately after withdrawal of the blood from the fish. By having the unit prepared in advance the total time between removal of the blood from the animal and reading of the sample was reduced to about 25 minutes. This analysis was also conducted at 10-12°C to prevent increased deamination due to a Q_{10} effect. Accuracy was improved also because the samples were not diluted. Under these conditions, without

TCA, lower blood ammonia values were obtained than before when TCA was used.

All the data presented here were obtained without use of TCA. Some deamination may have occurred, but this probably does not effect the significance of the relative changes in blood ammonia values observed for fish exposed to various concentrations of ammonia.

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