ENDOGENOUS NITROGEN METABOLISM

IN

ONCORHYNCHUS KISUTCH

by

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ABSTRACT

Reviews are presented of the pertinent literature dealing with endogenous nitrogen metabolism in both mammals and fish. In addition, the endogenous nitrogen excretion of the coho salmon, Oncorhynchus kisutch was investigated. Ammonia is believed to constitute the major nitrogenous waste product of fish. Therefore, the rate of ammonia nitrogen excretion by individual fasting fish in distilled water was quantified. The rate of elimination was found to increase with a rise in ambient temperature from five to twenty degrees Celsius. However the results obtained were exaggerated in magnitude. As an alternative procedure, the body composition of fingerling salmon was analysed during a ten week fast. Endogenous nitrogen was then correlated to the rate of protein oxidation and to heat production. The latter was determined from the rates of both protein and fat catabolism. A tentative value for endogenous excretion was determined - 23 milligrams nitrogen per kilogram fish per day. The ratio of rate of endogenous nitrogen excretion to calculated metabolic rate was estimated to be 2.3 milligrams excreted nitrogen per kilocalorie.

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INTRODUCTION

Man uses a complement of some twenty-two amino acids. Nine to twelve of these are essential in that they cannot be synthesized or their rate of synthesis is insufficient to meet metabolic needs. The food industry is primarily concerned with the provision of these compounds in the form of animal or vegetable proteins. Through the efforts of various international agencies such as the Food and Agricultural Organization of the United Nations, increased attention is being given to the contribution that the fresh and salt waters of the world can make to this required pool of high quality proteins. The efficiency with which fish are able to convert their various sources of ingested nitrogen to high quality proteins that might prove acceptable to the world food industry and hence to man must be investigated. The aim of this study was to enhance the present limited knowledge of the utilization of nitrogen and its biological compounds by fish. To this end, this study is most conveniently subdivided into three major sections: Part I; a review of the germane aspects of mammalian protein metabolism (nitrogen metabolism) to serve as a background for the experimental studies undertaken, Part II; a summary of the current knowledge of the protein metabolism (nitrogen metabolism) of fish and Part III; an account of attempts to establish the endogenous nitrogen excretion of a salmonid fish.

PART I: ENDOGENOUS MAMMALIAN NITROGEN METABOLISM

Modern concepts of proteins and their metabolism are rooted upon investigations in the early nineteenth century. At that time, rudimentary chemical analyses indicated that the animate world was formed of hydrates of carbon (starches, sugars), fats and quaternary azotized substances. The latter (egg albumins, plasma, casein, fibrin, wheat gluten for example) were distinguished by a high, relatively constant content of nitrogen. Thus typical studies of mammalian muscle showed the following composition: water 75 per cent, protein 18.5 per cent, soluble non-protein substances (such as carbohydrate, inorganic, phosphorus containing and nitrogenous compounds) 3.5 per cent and intracellular fat 3 per cent (Bate-Smith 1946).

Curious as to the role these products played in the life process, François Magendie found that a diet of sugar or fat in the absence of these albumins was unable to support life in a group of dogs (Beach 1948). Thus he realized that the nitrogenous compounds were vital to the maintenance of the living state. Later, Gerard J. Mulder in 1838, coined the term "protein" to signify the primary nature of these substances (Beach 1948).

As the fundamental chemical studies of the proteins progressed, degradation products were characterized and individual amino acids isolated. These researches were eventually incorporated into the building block theory of protein structure. However, at the same time the nitrogenous excretion products of the body were also investigated.

This work led to the identification of urea as the end product of body protein catabolism in mammals.

Some workers then became concerned about the extent of dietary food utilization. As a means of quantification, J. B. Boussingault in 1836, introduced the concept of nitrogen balance (Beach 1948). Working with milk cows, he found an equilibrium relationship between the nitrogen intake and nitrogen loss. The measurement of this nitrogen balance became an important tool to further understanding of protein nutrition. For example, Carl Voit among others was able to demonstrate that proteins from various sources differed in their usefulness to the animal body; that is, that all proteins were not the same (Beach 1948).

With some knowledge of the chemistry of proteins and of their nutritional importance, workers then questioned the role of protein in body chemistry. Justus von Liebig believed that the non-nitrogenous constituents of the diet supplied respiratory energy (Beach 1948). The proteins, he thought were directly assimilated into blood and tissue protein, the degradation of the latter supplying muscular energy. Voit refuted this idea by showing that exercise did not increase urea production. However, by using dogs fed at varying protein levels, he demonstrated a direct correlation with the amount of nitrogen excreted (Beach 1948). When protein intake was changed (either raised or lowered), a corresponding shift occurred in nitrogen output after an initial lag period. An equilibrium (or nitrogen balance) condition was achieved. Voit therefore concluded that there was a variable pool of labile body protein, the amount of which is related to the level of dietary protein. While this store is readily catabolized, tissue protein is resistant to oxidation. Folin (1905) attempted to develop

this theory by studying the effects of high and low nitrogen intakes on urinary composition (Mitchell 1962a). Noting that the output of urea and inorganic sulfate changed with protein intake, while the excretion of creatinine, neutral sulfur, uric acid and ethereal sulfates were affected little, if at all, Folin concluded that two separate forms of protein metabolism exist. The one which was variable and dependent upon diet, he classified as exogenous. The other which was constant, seemingly a product of metabolic processes indispensable for the continuance of life, he labelled endogenous. Schoenheimer's studies in the 1930's revealed a fallacy in these arbitrary classifications (Munro 1964a). With isotopically labelled amino acids (N^{15}) in a rat diet, it was evident that an interplay was involved. Once amino acids were in the body pool, rapid reactions resulted in interchange and incorporation of some N^{15} amino acids into tissue protein. Thus metabolism of dietary protein was not independent of the tissue protein reactions. Furthermore in some tissues, proteins were more labile (subject to degradation and reformation) than in others. The concept of amino acids being in a "dynamic equilibrium" was therefore postulated.

Protein metabolism is an all-inclusive term for the sum of the anabolic and catabolic reactions involving that group of high molecular weight, nitrogen-rich substances ubiquitous in biological systems. With such a comprehensive view, one could well acknowledge the demarcation of independent areas of investigation united by theme. In other words, from a historical perspective the study of proteins evolved into researches in pure biochemistry and into nutrition.

The intent here is not to attempt to review the field in all its permutations but to link certain findings concerning the utilization and excretion of proteins in the mammal with proposals of those mechanisms and their importance in fish. Much study has been given to the metabolism of these compounds in "higher" animals. It would be reasonable to expect that a number of predictions of value could be extended to the teleosts, to be tested by experimental analysis. The concern over the possible future role of fish in human nutrition justifies a basic experimental approach to their nutritional requirements.

Fundamental to a discussion of protein utilization, is a brief review of catabolism and excretion, work which has been detailed only relatively recently. In any biochemical text of the last ten years, one readily identifies proteins as a varied group of chemically related substances fulfilling a variety of tissue and cellular functions. They are synthesized as combinations of structural subunits called amino acids of which there are said to be twenty-four. These amino acids are on the average 16 per cent by weight of nitrogen. Therefore one of the most important methods of quantifying protein is by nitrogen analysis followed by multiplication by the conversion factor $\frac{100}{16}$ (6.25).

When dietary protein is ingested it is conveyed to a stomach well supplied with hydrochloric acid. The acid hydrolyzes the protein molecule to some extent resulting in a change in its three-dimensional configuration, exposing more of the amino acid peptide bonds to the medium. This "denaturation" of the molecule enhances enzymatic attack

which begins in the stomach and continues in the small intestine. The proteins, hydrolyzed to successively-smaller units are eventually absorbed across the intestinal epithelium and into the blood stream as amino acids. Transported to the liver which is the locus of supply and demand, the distribution point, the dietary amino acids mix with others produced by body protein catabolism. Their subsequent individual fate may follow one of several courses: some, small in proportion are excreted as such in the urine via the kidney, others are used for the synthesis of proteins or non-protein nitrogenous compounds, still others are deaminated, the carbon skeleton oxidized to yield energy or used for the synthesis of glycogen or fat (energy storage compounds).

Of particular interest to this discussion is the metabolic destiny of the amino group. In the parent amino acid molecule, the amino radical is the only component which can not be oxidized by animals. Unavailable for energy production, the toxic ammonia produced upon deamination poses a severe threat to continued cell function.

In the mammal, nitrogen is eliminated in one of three ways; first, urinary excretion; second, faecal waste; and third, surface (hair, skin, glandular secretion) losses which may be considered in most cases, as incidental to whole body metabolism. The first means reflects the degration of nitrogenous tissue compounds. Therefore, once nitrogen excretion via the kidney is indicated, it is necessary to state that a number of protective mechanisms handle the ammonia molecule.

One of the most important metabolic intermediates is α - ketoglutarate, a dicarboxylic member of the Krebs cycle. This compound

is converted to the amino acid glutamate via a transamination reaction with other α-amino acids. This then is one mechanism for the removal of amino groups in which free ammonia is not liberated. The enzymes which catalyze oxidative and non-oxidative deamination do produce free ammonia, which is thereafter bound by one of two means. The molecule may be removed from solutions by an amide linkage to glutamate forming glutamine. Hydrolysis of the latter compound is thought to be the primary source of ammonium ion in urinary acid-base balance (Goldstein and Forster 1970). However the most common route of elimination of protein nitrogen is as urea. Glutamate and other α-amino acids are deaminated releasing free ammonia. Carbamyl phosphate is then synthesized from the ammonia and carbon dioxide and combines with the first intermediate of the urea cycle, citrulline. Following a series of reactions, urea is eventually synthesized and eliminated as a by-product.

Having pursued proteins on a molecular basis or at the "test tube level", one can now approach the equally complex area of whole body nutrition. As is to be expected, when attempting to note the effect of a particular substance upon a living organism a great many variables prevail which must be monitored in order to obtain consistent results. The animal body may be likened to a black box which is continuously producing information dependent upon input. Within the box a multitude of chemical reactions are occurring which may be studied individually but whose ultimate effect is the result of highly dependent interactions. With that preamble, one can continue by stating that investigators have theorized about and experimentally established certain relationships derived from micro-observations and

interpreted on a macro-scale.

As stated previously, Voit provided the greatest stimulus to the detailed analysis of protein in nutrition using perfected nitrogen balance techniques (Beach 1948). This method of evaluation has proved highly useful to an understanding of the biological budgeting of proteins. The procedure basically involves measurement of the intake and output of protein as nitrogen. Thus while all dietary food supplies are sampled, faeces and urine are collected. By subtracting total body nitrogen losses from total intake one can determine whether the individual is in positive or negative balance - storing or discharging nitrogen. However before progressing further it must be stated that the amount of faecal nitrogen is related to the variability of protein digestion and absorption whereas urinary nitrogen is a product of body protein metabolism.

Working with dogs, Voit formulated a theory about the nature of body protein derived from the results of certain balance studies. The subjects were fed rations which varied in protein content and were then starved while urea-nitrogen was measured (Munro 1964b). Over a period of six days, nitrogen excretion rapidly diminished to reach an approximately constant level. Observing that the quantity excreted in excess of the minimum was proportional to the level of protein in the preceding diet, Voit concluded that there must be two types of body protein. One associated with essential structures is stable and not readily available upon metabolic demand. The other which is a labile form found in plasma and loosely bound to certain cells is easily mobilized. The concentration of dietary protein would determine the amount of the latter to be retained by the system. Under the most

favourable conditions for accumulation, Voit believed that the labile body protein stores did not exceed 5 per cent of the total carcass protein content. Later studies by other workers indicated figures of 3 per cent for humans and 0.5 to 3 per cent for rats (Munro 1964b).

The dual nature of protein metabolism which Voit envisioned, in the process of time was extended and experimentally verified. Of fundamental importance to these developments was cognizance of the intimate relationship of fat, protein and carbohydrate to the energy metabolism of the animal. The caloric requirements have been extensively studied in a wide variety of species. In order to obtain a reference point, both to enable inter-species comparisons and to evaluate the energy costs of a number of activities, a state of basal metabolism has been defined (Brody 1964). To achieve this condition of minimal energy expenditure specific standards must be observed:

1. a post-absorptive state - one avoids the energy expenditures involved with digestion, absorption and intermediary metabolism
2. a resting state - one eliminates the energy increment due to activity
3. a thermoneutral environment - with homeothermic animals a temperature zone exists beyond the limits of which, metabolic stress is imposed to either cool or warm the body. Within the comfort zone, a steady-state of minimal adjustment is maintained.

Much work has been devoted to the measurement of energy demand. The fuels of animal tissues are carbohydrates, fats and proteins whose total biological combustion consumes oxygen and produces carbon dioxide

and water. The caloric equivalent of a unit volume of oxygen varies with each substrate. In other words, carbohydrate oxidation results in the production of 5.047 kilocalories per litre of oxygen consumed, mixed fat oxidation, 4.69 kilocalories and mixed protein, 4.82 kilocalories. The range of maximum variation (4.69 to 5.05) is small (7 per cent) and the deviation from the mean value (3.5 per cent) is within the limits of experimental error (Brody 1964). Therefore, although the exact composition of the body fuel mix is not known, one can measure the amount of oxygen consumed, multiply by the mean caloric equivalent (4.825 kilocalories per litre oxygen) and so obtain a good approximation to the body energy release. When the subject is held under basal conditions one can then estimate the minimal caloric expenditure associated with the living state.

With such a useful tool, investigators attempted to draw a mathematical correlation between a body parameter and basal metabolic rate. Predictions of energy needs could therefore be made without the necessity of oxygen measurements. Many relationships were postulated, one of the more well-established being that of Brody (1964) and of Kleiber (1961).

$$Q = 70.5M^{0.734}$$

Q = basal metabolic kilocalories produced per day

M = body weight in kilograms

One could say that this equation signifies an average basal metabolism of seventy and one-half kilocalories per day per kilogram body weight raised to the 0.73 power for a mature homeothermic animal. Having determined the minimal energy needs associated with basal metabolism, one can then spare the combustion of body tissue by the provision of an

equivalent amount of dietary calories.

Voit and later Terroine first demonstrated that protein could be used as an alternative energy source. The former determined the urea excretion of dogs during starvation (Beach 1948). While there were ample body fat stores for oxidation, nitrogen excretion was relatively low. Upon depletion of this source, protein was degraded and nitrogen excretion increased. Terroine, Brenckmann and Feuerbach (1922) examined the body composition of normal and starved mice. As Table I indicates, fat content was markedly diminished, protein to a lesser extent.

TABLE I

BODY COMPOSITION OF NORMAL AND STARVED MICE

(TERROINE, BRENCKMANN AND FEUERBACH 1922)

Component	Normal (% Wet Weight)	Starved to Death (% Wet Weight)
Water	61-75	75-77
Ash	2-3	2-3
Organic matter	22-37	21-22
Protein (N X 6.25)	16-25	17-20
Fat	3-9	2.3-2.5

The continual expenditure of energy coupled with life, must of necessity flow from dietary sources if body tissues are to remain intact. If the total caloric needs are supplied by fat and carbohydrate one would expect that dietary protein could be regulated to replace only incidental metabolic losses. Therefore the minimal loss of nitrogen on a protein-free calorically sufficient diet can be equated with the minimal requirement. The reactions involving protein consumed in excess

of this amount or exogenous metabolism may be considered as the body's method of ridding itself of nitrogen not required for endogenous replacement and growth (Mitchell 1962b).

The channels of endogenous excretion are via the faeces and urine. Twombly and Meyer (1961) estimated that alimentary nitrogen output for a rat on a protein-free diet was equivalent to the amount consumed on a 10 per cent protein diet. Schneider (1934) has shown with the rat that the amount of faecal nitrogen excreted is proportional to the dry matter ingested. Since it is a quantity affected by roughage in the ration, minimal faecal nitrogen must be rechecked with each change in experimental design. The gut losses are comprised of mucoproteins, unabsorbed digestive enzymes and juices, mucosal cell sloughage and intestinal flora (Gitler 1964). This endogenous secretion increases with a protein diet. A linear relationship has been demonstrated between the ratio of faecal nitrogen to dry matter consumed and the level of dietary protein (Mitchell and Bert 1954).

A quantity which has been found to be far more significant and reliable is endogenous urinary nitrogen. Once restricted to a protein-free diet with the concomitant degradation of protein stores, a subject attains a fairly steady minimum level of nitrogen excretion. Smuts (1935) showed that depending upon the size of animal, the period required to reach the level of endogenous metabolism need not exceed fifteen to twenty days. Brody (1964), for example found that an interval of about four days was sufficient for the rat and man. Work with a variety of mammals and in different laboratories has indicated that endogenous nitrogen excretion bears a constant relationship to

basal metabolism. The figure which is commonly quoted is two milligrams of urinary nitrogen excreted per basal kilocalorie (Blaxter and Wood 1951; Bricker, Mitchell and Kinsman 1945; Mitchell and Beadles 1950; Smuts 1935; Treichler and Mitchell 1941). The significance of this relationship must not be over emphasized nor construed to be an independent function. The metabolic significance is not clearly understood. However the constancy of this ratio in terms of minimal requirements provides an extremely useful reference.

Using the data of independent researchers, Brody (1964) examined the relationship between body weight and endogenous urinary nitrogen excretion of mature animals of different species. The equation which he fitted to their results bears a distinct resemblance to that for metabolic rate.

$$N = 146\ M^{0.72}$$

N = milligrams endogenous urinary nitrogen excretion per day

M = body weight of mature animals in kilograms

The body weight exponents (0.72 and 0.73) are not significantly different. Therefore the ratio of nitrogen excretion to basal metabolic rate (146/70.5) is again approximately two milligrams per kilocalorie.

The determination of this quantity, endogenous urinary nitrogen is fraught with certain difficulties. For example, the accuracy and precision of evaluation is dependent upon the duration of nitrogen-free feeding prior to the collection period. In other words, the measure hinges upon the magnitude of the body's labile protein stores which varies with previous protein intake.

A further consideration is the distribution of nitrogenous end products in the urine. The constituent which was found to vary with protein intake was urea. Creatinine and to a smaller extent, uric acid and ammonia comprise the endogenous constant fraction. The effect of dietary protein intake on the variable and constant nitrogen moieties is illustrated in Table II. The excretion of urea as can be seen is most particularly affected. Protein-free feeding in dogs (Table III) results in a decrease in urea nitrogen with time to a minimal level accompanied by a relatively fixed endogenous excretion of ammonia and creatinine. Blaxter and Wood (1951) suggested as a result of studies on calves, that 12 per cent of endogenous metabolism involves the irreversible reaction creatine to creatinine, 25 per cent purine catabolism (allantoin, uric acid) and 50 per cent reactions terminating in the excretion of urea and ammonia.

TABLE II

PARTITION OF URINARY NITROGEN OUTPUT BY ADULT HUMAN SUBJECTS (FOLIN 1905)

Urinary Nitrogen	High Protein Diet		Low Protein Diet	
	Total Daily Output (gN)	Per Cent of Total Nitrogen	Total Daily Output (gN)	Per Cent of Total Nitrogen
Total N	16.80	100	3.60	100
Urea-N	14.70	87.5	2.20	61.2
Ammonia-N	0.49	2.92	0.42	11.7
Uric acid-N	0.18	1.07	0.09	2.5
Creatinine-N	0.58	3.45	0.60	16.7
Undetermined-N	0.85	5.06	0.27	7.5

TABLE III

URINARY NITROGEN EXCRETION OF DOGS ON A PROTEIN-FREE DIET (ALLISON AND BIRD 1964)

Weeks of Protein Depletion	Urea-N * (mg/day/kg body weight)	Urinary * Ammonia-N (mg/day/kg body weight)	Creatinine * (mg/day/kg body weight)
1	162	22.5	20.4
2	111	22.5	21.0
3	94	22.1	21.4
4	93	21.5	19.3

* averages of data from a study of sixteen dogs.

Protein metabolism may be subject to yet another physiological stress - starvation. There are two ways in which the effect may be assessed; the one is an analysis of body composition, the other is an examination of nitrogenous waste coupled with measurement of total energy metabolism. As stated previously, the animal body has a continual need for fuel. The potential energy contained in the molecules of dietary organic foodstuffs is released by oxidation and trapped in the "high energy" phosphate bonds of adenosine triphosphate (ATP). The hydrolysis of ATP provides the impetus enabling otherwise thermodynamically unfavourable reactions to proceed to completion. When exogenous energy sources are not available, tissue stores of carbohydrate, fat and protein are utilized. The extent of combustion over a narrowly defined time interval should give a reasonable estimation of minimal exogenous protein, calorie requirements.

A preliminary gross approach to the subject is an examination of the changes in body composition with time. The study of Farrell and

Reardon (1972) provides a good example of what can be expected upon semi-starvation. A group of twenty-four sheep had been divided into three groups. Group A was well-nourished while groups B and C were undernourished for four months and then maintained in a thin condition for another nine months. The sheep were then all killed and analysed. There were significant differences. On a fat-free body weight basis, protein content decreased while water content increased with undernutrition. Body fat was also markedly diminished with severe restriction of diet.

Terroine and Synephias (1937) give figures indicating the relative contributions of proteins towards the energy production during starvation. They found that the proportion seemed to vary with species: 30 per cent for the rabbit, 15 per cent in man and 6 to 10 per cent in the pig, mouse and rat.

These conclusions may be compared with those of Benedict and Fox (1934) who measured nitrogen excretion in the fasting rat (Table IV). As indicated, prolonged fasting resulted in a marked decrease in metabolic rate and an increased level of protein combustion as fasting progressed. The proportion of calories derived from protein oxidation increased from 13 to 25 per cent over ten days.

The relation of these figures to a normal physiological state is a point of contention. To reiterate, a mature homeothermic animal excretes about two milligrams endogenous urinary nitrogen per basal kilocalorie. When adequate supplies of fats and especially carbohydrates are available, proteins rank in tertiary importance as an energy source (Munro 1964b). Therefore since glycogen stores in the body are insignificant and rapidly diminished upon initiation of

fasting, the relationship of protein degradation during starvation to that minimal amount under normal conditions is disputable.

TABLE IV

PROTEIN AND ENERGY METABOLISM OF RATS DURING PROLONGED FASTING (BENEDICT AND FOX 1934)

Days of Fast	Body Weight (g)	Metabolic Rate (A) (kcal/kg/day)	Nitrogen Excretion (B) (mg/kg/day)	Ratio of B to A (mg/kcal)	Per Cent of Total kcal Derived from Protein Oxidation
1	121	144.5	704	4.87	13
7	96	122.0	728	5.97	14
10	84	92.5	864	9.34	25

In summary, studies on the endogenous nitrogen excretion of mammals have indicated a rather constant relationship to basal metabolic rate. Two milligrams of endogenous urinary nitrogen are excreted per basal kilocalorie of heat produced. The above association has been experimentally tested in mature homeothermic animals of a wide variety of species and has since become a traditionally accepted reference value (Brody 1964; Smuts 1935; Terroine and Sorg-Matter 1928).

PART II: ENDOGENOUS NITROGEN METABOLISM IN FISH

Although mammalian protein and energy metabolism has been extensively examined, workers have given limited attention to similar investigations with fish. Since conclusive evidence in many areas is unavailable, facts give way to conjecture. With the assumption that a unity of biochemical form and function exists among animals (Lehninger 1970) and aided by certain experimental observations one can draw correlations with known systems and attempt to predict responses to be tested by bioassay.

First, before proceeding with a more detailed discussion, it is imperative to recognize two fundamental attributes of the class Pisces or more especially the suborder Teleostei, the true bony fishes. They are poikilothermic and they survive in water. While this statement appears elementary, one must remember that comparisons are to be drawn with terrestrial homeotherms subject to rather different physiological stresses.

Changes in environmental temperature as reflected by body temperature would therefore be expected to have an important influence on metabolism. The rate of a biological reaction is a function of temperature, described by the Arrhenius equation:

$S = A\, e^{kt}$ (Brody 1964)

S = rate of process at temperature t

A = empirical constant

k = the differential increase in relative rate of change for a 1°C. change in temperature

or taking natural logarithms,

$$\ln S = \ln A + k\,t.$$

Reworded, for a temperature increase over a narrow range, the rate of an enzyme catalyzed reaction increases. Therefore, since a biological system is a medley of enzyme reactions, one could expect an increase in rate of metabolism with a rise in ambient temperature. However since enzymes are proteins they are also subject to degradative changes at certain temperature limits with a concomitant loss of activity. In consequence, for a given system one expects to find a temperature optimum that is most favourable to rapid development.

The second arresting feature of teleosts is a dependence upon water. The complex organization of living body cells requires mechanisms of respiration and excretion adapted to a water medium in order to survive. Energy metabolism as for mammals necessitates an exchange of oxygen and carbon dioxide. In the fish this function is performed by the gill. Oxygen, continuously stripped from solution is absorbed across the epithelium. Catabolism may therefore be monitored simply by measurement of the concentration of dissolved oxygen in the ambient water. Excretory adaptations present a novel situation.

At this point one must recognize ureotelism as predominantly a terrestrial adaptation and ammoneotelism as an entirely aquatic one. Ammonia is known to be a highly toxic compound to animal cells. For example, if blood ammonia reaches a concentration of fifty micrograms per millilitre (50 µg/ml) in rabbits, death ensues (Baldwin 1967). However it is also the by-product of protein metabolism. Removal from or detoxification in the body is therefore of primary importance. Since ammonia is readily soluble in water as the ion, tissue dilution

of ammonium ion and excretion can be realized if considerable quantities of water are available. Since fish experience a constant flux of water over the gill epithelium one finds that ammonia is readily eliminated and constitutes the major fraction of nitrogenous excretory products. Teleosts are consequently classified as ammoneotelic (Baldwin 1967).

On the other hand, where water is a scarcity and is carefully harboured, ammonia can not be excreted as such. Within mammals then, one discovers a mechanism of detoxification of ammonia via synthesis and subsequent excretion of urea. These species are denoted as ureotelic (Baldwin 1967).

Unlike mammalian elimination, the biochemistry of nitrogen excretion in fish has not been extensively examined. Analysis of the nitrogenous waste of these classes has indicated that although there is a similarity of composition, there is a marked difference in distribution. Despite probable numerical modifications due to species differences among teleosts, workers have found that approximately 80 per cent of the total metabolized nitrogen excretion occurs via the gills. The remainder is voided through the kidney (Smith 1929; Wood 1958). In addition it has been well established that ammonia comprises the bulk of excreted nitrogen (Delaunay 1931; Denis 1913; Furukawa and Ogasawara 1955; Smith 1929; Smith 1930; Wood 1958) (see Table V). Wood (1958) found in some marine teleosts that ammonia and urea together accounted for 75 to 98 per cent of the total excreted nitrogen, the ammonia fraction being some 50 to 80 per cent and urea comprising 20 per cent of the total nitrogen. A large part of the variability observed in the magnitude of the ammonia fraction is due to the non-constant "undetermined non-protein nitrogen" fraction. In

TABLE V

PERCENTAGE COMPOSITION OF NON-PROTEIN NITROGEN EXCRETION IN TELEOSTS

Animal	Reference	Ammonia N*	Urea N	Urate N	Creatinine N	Creatine N	Amino Acid N	TMA** N	TMAO*** N	Other N
Freshwater										
Carassius	Prosser (1950)	73.3	9.9	-	-	-	-	-	-	16.8
Cyprinus	Ibid.	59.7	6.2	0.2	-	-	6.5	-	-	22.0
minnow	Ibid.	77.4	14.5	-	-	-	2.6	-	-	-
Cyprinus carpio	Smith (1929)									
via gills (% of total)		62.0	6.0	-	-	-	7.8	-	-	15.2
via urine (% of total)		1.5	0.7	0.2	0.0	4.4	1.7	-	-	0.7
Marine										
starry flounder	Wood (1958)	86.2	11.5	-	← 0.3**** →		-	1.1	0.5	0.4
sculpin	Ibid.	52.6	22.5	-	← 0.4**** →		-	0.0	2.2	22.4

* nitrogen
** trimethylamine
*** trimethylamine oxide
**** total combined figure for creatinine and creatine

marine species, a major portion of this amount is said to be trimethylamine oxide (TMAO) (Forster and Goldstein 1971). As to the source of TMAO, controversy rages. Some workers believe that the compound is a product of endogenous synthesis (Goldstein, Hartman and Forster 1967) while others believe the level of excretion to be dependent upon dietary intake (Benoit and Norris 1945; Wood 1958).

Although ammonia is known to be eliminated via the gills, the source and mechanism of formation has been but tentatively investigated (Goldstein and Forster 1963; Goldstein, Forster and Fanelli 1964; Janicki and Lingis 1970; Makarewicz and Zydowo 1962; Pequin 1962).

The synthesis of urea, again poses an enigma. The urea cycle, the detoxifying mechanism of mammals does not function to a significant extent in teleosts (Brown and Cohen 1960; Huggins, Skutsch and Baldwin 1969). Arginase, the enzyme of the urea cycle which catalyzes the synthesis of urea has been found only in low concentration in the livers of cod, trout, bullhead and salmon (Hunter 1924). The alternate mode of synthesis as proposed by Goldstein and Forster (1965) is the degradation of the purine bases of nucleic acids to uric acid and subsequent conversion to urea. Whatever the synthetic mechanism involved, it appears that marine teleosts, in a hyperosmotic environment requiring a more restricted elimination of water may excrete more urea than freshwater species (Prosser 1950).

Upon reflection, one could certainly expect the protein metabolism of fish to be similar to that of mammals. Since the biochemical composition of living organisms is basically uniform, one envisions analagous approaches to food utilization. In other words, one can determine the endogenous or maintenance protein requirements of

fish using techniques employed in mammalian studies. Thus analysis of the total nitrogen excretion over a controlled period on a nitrogen-free, calorically adequate diet would yield a value for minimal protein demand.

However, as well as a lack of exact analyses of the composition of fish nitrogen excretion, there are only sparse reports on the relationship between protein consumption and nitrogen waste in the literature. To the writer's knowledge only two workers have previously attempted to actually measure the endogenous nitrogen excretion of fish. Gerking (1955) was the first to publish a value for the endogenous nitrogen excretion of the bluegill sunfish. The fish, kept at an average temperature of 25.9 degrees Celsius (°C.) were supplied daily with glucose. This sugar, which Phillips, Tunison and Brockway (1948) indicated trout could assimilate was provided as an energy source in an amount judged sufficient to meet daily metabolic expenditure. Gerking believed that the nitrogen excreted by the fish on this diet could be equated with endogenous nitrogen excretion. After following the nitrogen output of fish of different weight over a period of five days, Gerking calculated a regression curve:

$$\log y = -0.0282 + 0.5394 \log x$$

y = nitrogen excreted in milligrams per day

x = beginning body weight in grams

Thus for a body weight range of twenty to thirty grams, the ratio milligrams nitrogen excreted per gram per day varies from 0.24 to 0.20. As body weight increases, nitrogen excretion per unit body weight diminishes. In addition, having measured the metabolic rate of the

bluegill, Gerking estimated that about seven milligrams of endogenous nitrogen are excreted per kilocalorie. This ratio should be compared with the figure of two milligrams nitrogen per basal kilocalorie derived from mammalian data (Brody 1964). The large discrepancy between these results could indicate that fish metabolize protein to a greater extent for energy or are more inefficient in recycling body protein. Or, it could also be a by-product of the techniques employed in the measurement of nitrogen excretion and metabolic rate as well as in the handling of the fish. On the other hand, the excreted nitrogen to basal kilocalorie ratios for some poikilotherms may be rather large. Bonnet (1933) studied the nitrogen excretion and metabolic rate of the frog and turtle at different temperatures. He found a ratio of 7.3 for the frog at 15°C. and 2.5 milligrams nitrogen per kilocalorie for the turtle at 20°C.

Savitz (1969) also worked with the bluegill sunfish. In the wake of Gerking's example, Savitz studied the nitrogen output of individual fish on a glucose diet. Besides examining subjects of different body weights, Savitz also determined the effect of temperature variation. Table VI lists the regression equations which he calculated for the relationship between body weight and the rate of nitrogen excretion for fish held at four temperatures. As shown, the weight exponent varies from 0.99 to 0.96. Compare these figures to the exponent 0.73 as computed for mammals (Brody 1964). Perhaps one should state here, that Savitz does not make clear in his paper whether he is measuring endogenous metabolized nitrogen exclusively. His sampling technique did not preclude faecal nitrogen. If total nitrogen output were being measured, this too would be erroneous since no steps were

TABLE VI

LINEAR EQUATIONS RELATING BODY WEIGHT TO NITROGEN EXCRETION RATE IN THE BLUEGILL SUNFISH (SAVITZ 1969)

Temperature (°C.)	Number of Fish	Linear Equation	SE
29.4-32.2	15	$\log y^* = -0.52550 + 0.99063 \log x^{**}$	±0.075
23.9	40	$\log y = -0.89079 + 0.97656 \log x$	±0.101
15.6	14	$\log y = -1.17675 + 0.93138 \log x$	±0.300
7.2	20	$\log y = -1.17483 + 0.95979 \log x$	±0.160

*y = nitrogen excretion in milligrams per day

**x = body weight in grams

taken to ensure homogeneity of a faecal suspension. Nevertheless, Table VII shows plainly that temperature does play a role in the level of metabolism and hence rate of nitrogen excretion. Elevated temperatures lead to increased nitrogen excretion. Schütz (1912) found with fasting tench that nitrogen metabolism may vary 11.4 per cent per degree centigrade. The results presented by Savitz indicate that the change is not constant. Also, it is evident that the fish of lower body weight exhibits a higher rate of nitrogen excretion per unit weight (as Gerking (1955)). This result may emphasize the differences to be expected in metabolic activity at a range of "physiological ages" or body weights.

An important consideration in terms of intra- and inter-species comparisons of nitrogen elimination is "physiological age". If one were to study the growth of animals from birth to maturity, one would be scanning a discontinuous process. In other words, the instantaneous rate of growth is not constant. A plot of weight versus

TABLE VII

MEAN NITROGEN EXCRETION RATES FOR BLUEGILL

SUNFISH CALCULATED FROM TABLE VI

(SAVITZ 1969)

Temperature	Subjects			
	100g fish		10 g fish	
(°C.)	Mean N* Excretion Rate (mg/day)	95% Confidence Limits	Mean N* Excretion Rate (mg/day)	95% Confidence Limits
29.4-32.2	28.56	23.18-35.20	2.92	2.60-3.27
23.9	11.54	9.46-14.08	1.21	0.94-1.58
15.6	4.85	2.26-10.43	0.57	0.22-1.46
7.2	5.55	3.98-7.76	0.61	0.40-0.90

*nitrogen

time would yield at first glance, a sigmoid curve. Naturally for a given age interval, individuals of various species will be placed at different points of their growth curves, some animals attaining maturity earlier than others. However, all species appear to exhibit at one stage of development, a rapid increase of growth rate to a maximum after which a deceleration occurs (Brody 1964). Since a growing animal is highly dependent upon a diet adequate in energy, proteins, minerals and vitamins for optimum development, endogenous nitrogen excretion is a difficult assessment at this stage. Nonetheless one must be aware of this additional age factor when studying the protein metabolism of a species.

Coupled closely to protein requirements is energy metabolism. As mentioned previously, proteins, fats and carbohydrates are the fuels

of the life torch. The combustive degradation of each supplies a certain share of the total energy turnover. Thus for example, a diet can be geared to supply a quantity of proteins, fats and carbohydrates sufficient to meet minimal energy needs plus increments for the metabolic costs of activity, growth, egg production et cetera.

One of the conditions requisite to the determination of basal metabolism in the homeotherm was a state of rest. However, an aquarium fish is never completely inactive since fin and body movements continuously adjust and maintain position. This difficulty prompted use of the term "standard metabolic rate" for fish studies. This phrase signifies the nearest attainable approximation to basal metabolism (as defined by Brody (1964)) when all organs are at minimal activity (Fry 1957; Krogh 1914). Two additional levels of oxygen consumption or metabolic rate have also been defined for fish: a routine rate, when all movements are apparently spontaneous and an active rate which permits the highest continued level of activity.

Unfortunately, the measurement of energy metabolism in fish has received scant attention. Once again, experiments with aquatic poikilotherms impose certain restrictions. Oxygen consumption, as a parameter of body catabolism can be readily determined by the change in concentration of dissolved oxygen in ambient water. Normally the uptake of one litre of oxygen is associated with the generation of 4.82 kilocalories when protein is catabolized (Brody 1964). This figure is based upon the excretion of protein nitrogen as urea. However in the fish, this nitrogen is eliminated as ammonia releasing additional carbon for oxidation. Therefore protein combustion in fish may result in a greater energy release than hitherto considered.

As with mammals, progressive increases in body size and activity are reflected in an elevated heat production (Beamish 1964a; Wohlschlag and Juliano 1959). However, the relationship between metabolic rate and body weight differs from the mammalian case. For goldfish, Wohlschlag and Juliano (1959) found a body weight exponent of 0.85 as compared to 0.73 for mammals (Brody 1964). Beamish (1964a) studying brown trout, brook trout, sucker, bullhead and carp found exponents varying from 0.86 to 1.0. These results merely suggest that metabolic rate may be a more direct function of body weight in fish. On the other hand, Brett (1964) reported that in yearling sockeye salmon there was no significant correlation between metabolic rate and body weight.

Additional considerations are the effects of temperature and the partial pressure of dissolved oxygen on metabolism. Both Beamish (1964a) and Basu (1959) found that a decrease in the partial pressure of dissolved oxygen lowered the active metabolic rate. Furthermore, although standard oxygen consumption remained approximately constant down to a partial pressure of eighty millimetres mercury, below this, the standard metabolic rate first increased to a maximum and then with further reduction, decreased. As would be expected, temperature plays a crucial role in determining the level of oxygen consumption. Increase in ambient temperature elevates metabolic rate in fish (Basu 1959; Beamish 1964a; Brett 1964; Brett 1971; Peterson and Anderson 1969). Brett (1964) studied this phenomenon in yearling sockeye salmon at temperatures from 5° to 24°C. and derived the following:

$$\log (y - 36) = 0.48 + 0.071\ x$$

y = standard oxygen consumption in milligrams oxygen per kilogram per hour

x = temperature in degrees Celsius

Thus between 12° and 14°C., a yearling sockeye salmon may be expected to have a standard oxygen consumption between 57.5 and 65.8 milligrams oxygen per kilogram per hour. A quick calculation would indicate what these figures mean in terms of calories per day. At 12°C.:

(A) 57.5 mgO_2/kg-hr ≡ 1.80 m moles O_2/kg-hr

At standard temperature and pressure, one mole of gas occupies 22.4 litres.

Therefore (A) ≡ 40.3 mlO_2/kg-hr.

The caloric equivalent of oxygen is 4.82 kilocalories per litre oxygen.

Therefore (A) ≡ 0.194 kcal/kg-hr.

The standard metabolic rate of the salmon would be 4.66 kilocalories per kilogram per day. In comparison the total energy expenditure of a mature homeotherm would be 70.5 kilocalories per kilogram per day (Brody 1964).

These results were modified when Brett (1971) published another paper in which various biological parameters were followed with temperature. Both standard and active metabolic rates of sockeye salmon were measured from zero to 25°C. Graphs of rate of oxygen consumption versus temperature were presented from which a few values were taken and listed in Table VIII with corresponding calculated standard heat productions.

Having obtained an approximation to the standard metabolic rate or total energy consumption over a defined time period, one could then determine the ratio of endogenous excreted nitrogen to standard kilocalories as Gerking (1955) attempted to do. However, for reasons

TABLE VIII

STANDARD METABOLIC RATE OF THE SALMON

ONCORHYNCHUS NERKA AT DIFFERENT

ACCLIMATION TEMPERATURES (BRETT 1971)

Temperature (°C.)	Oxygen Consumption			Energy Metabolism	
	mg/kg fish/hr	m moles /kg fish /hr	ml/kg fish/hr*	kcal /kg fish /hr**	kcal /kg fish /day
5	45	1.41	31.6	0.152	3.65
10	55	1.72	38.5	0.186	4.46
15	70	2.19	49.0	0.236	5.66
20	110	3.44	77.0	0.371	8.90

*22.4 litres per mole at standard temperature and pressure

**caloric equivalent of oxygen is 4.82 kilocalories per litre (Brody 1964)

discussed in Part III, the measurement of metabolic (versus faecal inclusion) nitrogen excretion during maintenance on a nitrogen-free, calorically adequate diet poses special difficulties. Therefore, while aware that a dichotomy may exist by which the two analyses may not be equated, attention has been directed towards determination of endogenous nitrogen excretion during a fast. This evaluation may be approached in two ways; one, a quantification of nitrogen waste in ambient water and two, changes in total body protein content with time of starvation.

A lack of dietary calories necessitates a mobilization of body energy reserves. Carbohydrate storage in most animals is negligible (Swift and Fisher 1964). Therefore, one would expect the oxidation of tissue fat and protein to supply energy. The resultant changes in body

composition could then be followed in time by analysing individuals from a large homogeneous population. One could calculate the percentage loss in fat and protein and thus the respective weight losses per one hundred grams of fish. Since as a general rule, combustion in the body of one gram of fat releases about nine kilocalories and one gram of protein, four kilocalories (Brody 1964), the relative magnitude of protein oxidation (or nitrogen elimination) to total energy metabolism could then be determined.

The primary source of body composition data for fasting fish is from studies conducted during a spawning migration. Naturally under these stressed conditions, in time, severe depletion of both fat and protein occurs. The relationship of the protein oxidation here to a more normal state is debatable. As indicated in Table IX and other papers (Bilton and Robins 1971; Davidson and Shostram 1936; Morgulis 1919; Parker 1966; Schütz 1912; Storer 1967; Tomlinson, McBride and Geiger 1967), when fish are starved there are percentage decreases in both fat and protein and an increase in water content. Yet one can not easily compare the data of these several studies. Although total amounts of both fat and protein oxidized can be calculated, one can not determine the average daily metabolism since the sampling time spans are not given. As a result, only the general trends in body composition changes are useful.

Pentegov, Mentov and Kurnaev (1928) alone, working with the migrating Oncorhynchus keta salmon mention energetics. They reported that the proportion of the expenditure of fat energy to protein energy at first increases but then decreases in time. Upon the combustion of

one-third of the common energy reserve, proteins are oxidized in greater amount in relation to fat.

TABLE IX

BODY COMPOSITION CHANGES IN FASTING FISH DURING THE SPAWNING MIGRATION

Reference	Component	Percentage Composition of Wet Weight	
		Initial	Final
Greene (1919)	Fat	15	2.24
	Phosphorized fat	1.18	0.44
	Protein	20	14
	Water*	74.8	81.5
Kukucz (1961)	Fat: male	17.04	4.05
	female	13.82	2.85
	Protein: male	20.26	16.62
	female	20.53	17.30
Pentegov, Mentov and Kurnaev (1928)	Water: male	68.65	85.73
	female	66.95	84.63
	Fat: male	9.19	0.17
	female	11.28	0.49
	Protein: male	21.08	13.26
	female	20.68	13.96
	Ash: male	1.08	0.84
	female	1.09	0.92

*fat-free basis

One group did examine the effects of starvation, on the chemical composition of brook trout, under more controlled conditions (Table X)(Phillips, Livingston and Dumas 1960). These experiments are discussed at greater length in Part III in conjunction with this study.

Whole body analysis for protein during starvation does yield some indication of the degree of protein combustion and hence dietary replacement demand. However carcass analysis provides information only over a prolonged period of time during which changes in external or

TABLE X

EFFECT OF STARVATION ON THE BODY COMPOSITION OF BROOK TROUT

(PHILLIPS, LIVINGSTON AND DUMAS 1960)

Water Temperature 8.2°C.

Length of Starvation (weeks)	Per Cent of Wet Body Weight Water* (%)	Protein* (%)	Fat* (%)	Ash* (%)
0	79.7	11.7	5.4	1.54
1	80.0	10.9	5.1	1.62
2	78.9	11.9	5.3	1.87
3	79.3	11.2	4.7	1.89
4	79.6	12.2	4.9	2.22
5	78.3	11.6	4.6	2.33
6	81.3	10.8	4.2	2.11
7	79.5	11.1	4.7	2.41
8	80.9	11.5	4.0	2.39
9	81.1	11.7	3.8	2.51
10	78.9	12.0	4.3	2.61
11	81.2	11.0	3.8	2.67
12	83.5	10.3	3.4	2.42
	Water Temperature 14.4°C.			
0	79.7	11.7	5.4	1.54
1	79.6	11.2	5.0	1.76
2	80.0	11.5	5.1	1.82
3	80.4	11.9	4.5	2.09
4	77.2	13.8	5.0	2.53
5	79.5	12.2	4.5	2.43
6	82.0	10.2	3.7	2.24
7	85.0	8.5	2.6	2.13
8	82.3	10.4	2.9	2.75
9	83.4	9.1	2.4	2.79
10	82.0	9.7	3.2	2.97
11	84.9	8.8	2.6	2.82
12	85.9	8.4	1.7	3.13

*each result represents an average of two samples.

internal variables may unduly influence results. More importantly fish must be killed, thus introducing a discontinuity of design, a variation due to individuals. In addition several weeks are required for a definite trend to develop, coarsening the picture, eliminating the fine

detail. Nevertheless these studies are extremely useful to provide a continuous graph of several components simultaneously. Mathematical interpretation of this data may indicate certain "causal" relationships of great potential use. For example, the body composition studies of Groves (1970) on young non-fasting sockeye resulted in the following equations:

$$W = 0.00571\ L^{3.118}$$

$$P = 0.204\ W^{1.038}$$

$$FFDM = 1.113P$$

W = total body water (g)

L = fork length (cm)

P = body protein (g)

FFDM = fat-free dry material (protein and ash) (g)

With these tools at hand one could readily determine body composition of living fish checking growth merely by anaesthetization and measurement of length and weight. Even so, a more sensitive monitor of protein requirements would be quantification of daily nitrogen excretion.

The original objective of the present work was to determine the endogenous nitrogen excretion of a salmonid fish maintained under well-specified conditions. Having established that the end product of protein metabolism is nitrogen excreted primarily as ammonia, one must determine whether the early stages of starvation approximate an endogenous state. Daily quantitative examination of excreted nitrogen should, as shown in mammalian studies reveal a region of minimum constant output.

The most thoroughly documented study of this kind to date is one conducted by Fromm (1963). He examined the rainbow trout Salmo

gairdneri kept in 12° to 14°C. tap water. Over a fourteen day period of starvation, he found a rapid drop in nitrogen excretion during the first six days. This was accompanied by a similar drop in ammonia-nitrogen excretion. Yet during the rest of the period, the total waste nitrogen and ammonia-nitrogen measured were less variable. The data collected by Fromm and other workers is given in Table XI for comparison.

TABLE XI

MEASUREMENTS OF THE DAILY NITROGEN EXCRETION OF DIFFERENT FISH SPECIES

Reference	Fish Type	Temperature (°C.)	Total Nitrogen Excretion (mg/kg/day)	Ammonia-nitrogen Excretion (mg/kg/day)
Fromm (1963)	rainbow trout	12-14	136	76-83
Gerking (1955)	bluegill sunfish	25.9	38.9*	
Pora and Precup (1962a, b)	carp	19	348	
Smith (1929)	carp and goldfish	18.5	42 (96)**	

*calculated from a regression equation

**average figure for eight other fish

A restriction to the value of starvation data, is the effect of this condition on standard and routine oxygen consumption. Metabolic rate during a fast was found to decrease rapidly to a minimum within two to three days after which it remained approximately constant (see Table XII) (Beamish 1964b). Such a reduction implies a

TABLE XII

EFFECT OF STARVATION ON STANDARD OXYGEN CONSUMPTION OF FISH (BEAMISH 1964b)

Fish Type	Temperature (°C.)	Oxygen Consumption ($mgO_2/kg/hr$) Initial	Constant Starvation Level	Constant Level Reached After (Days)
Brook trout	10	75	50	3
White sucker	10	40	20	3
White sucker	20	120	75	3
White sucker	10	75	40	10
White sucker	10	45	35	2

concomitant decrease in the activity of protein metabolism. However as indicated by the work of Benedict and Fox (1934) with rats (Table IV), the fasting metabolic rate may decrease yet protein combustion becomes increasingly important as an energy source. Therefore the ratio of nitrogen excretion to basal metabolic heat production does not remain constant and in fact increases. Similar data has not been published for fish but one could hypothesize an analagous effect.

Undoubtedly one must regard all earlier quantitative studies on the nitrogenous excretion of fish with some misgivings. As previously emphasized ammonia is toxic and as such is tolerated in the blood to a very limited extent. In mammals the concentration of blood ammonia probably does not exceed one to three micrograms per millilitre

(Everett 1946). In frogs, reptiles and fishes it is less than one microgram per millilitre (Prosser 1950). Workers have thought that teleosts can readily excrete ammonia merely by passive diffusion across a concentration gradient or by a cation exchange mechanism (Maetz and Romeu 1964). However limitations were recognized. Exposure to excessive ambient concentrations of ammonia resulted in adverse effects on growth, fusion of gill lamellae diminishing the oxygen supply (Burrows 1964; Kawamoto 1958; Koerting 1969; Reichenbach-Klinke 1967). Postulated tolerance limits for certain species are listed in Table XIII. The actual analytical difficulty encountered in establishing nitrogen excretion rate values is the fine concentration balance required to measure excreted ammonia on the one hand, and on the other to eliminate any inhibitory effect on the fish.

TABLE XIII

TOXICITY OF AMMONIA TO FISH

Reference	Fish Type	Tolerance Limit (ug NH_3/ml ambient water)	
		15°C.	23°C.
Koerting (1969)	trout eggs	< 0.2	
Ibid	carp	2.0	
Burrows (1964)	salmon	< 0.7	
Brockway (1950	trout	< 0.3	
Wuhrmann, Zehender and Woker (1947)	-	0.1	
Hazel, Thomsen and Meith (1971)	striped bass		
	fresh	2.8	1.9
	brackish	2.8	2.1
	seawater	2.0	1.5
	stickleback		
	fresh	2.1	1.8
	brackish	5.2	2.4
	seawater	10.4	2.3

Some workers have found that an increase in the level of ambient ammonia results in a corresponding decrease in amounts of excreted ammonia and total waste nitrogen (Fromm and Gillette 1968; Olson and Fromm 1971). Fromm and Gillette (1968) placed trout in tap water solutions of ammonium chloride at 12° to 13°C. The concentrations ranged from zero, one, three, five to eight micrograms total ammonia per millilitre. After twenty-four hour exposure, the mean blood levels for total ammonia showed a direct correlation with the level of ambient ammonia and increased from thirty-eight to seventy-one micrograms per millilitre. Fish kept in solutions of zero to one microgram per millilitre ammonia showed mean blood levels of 0.6 to 1.3 micrograms per millilitre. The per cent of total waste nitrogen excreted as ammonia decreased from 52 in ammonia-free water to 30 in eight micrograms ammonia per millilitre water. Fromm concluded that since blood ammonia concentration always exceeded the water level, the increases in blood ammonia must have resulted from an inhibition of ammonia excretion rather than an inward transfer against a concentration gradient.

To recapitulate, knowledge of the endogenous nitrogen metabolism of the fish poikilotherm is very limited. Studies have indicated that the rate of excretion is dependent upon both temperature and body weight. However quantitative inter-species comparisons are not currently feasible. Only one worker (Gerking 1955) has presented a relationship between endogenous nitrogen excretion and energy

metabolism; that is, seven milligrams of endogenous nitrogen are said to be excreted per kilocalorie.

PART III: AN ACCOUNT OF ATTEMPTS TO MEASURE THE ENDOGENOUS NITROGEN EXCRETION OF A SALMONID FISH

Current quantitative information regarding the rate of endogenous nitrogen excretion (or protein replacement requirements) of fish is limited. Since the examination of any aspect of fish metabolism involves an unknown and highly complex system, this study approached the subject in as simple and controlled a manner as possible. Consequently the experimental work was divided into three investigations:

1. an analysis of the ammonia nitrogen excretion of coho salmon in distilled water at 11° to 12°C.
2. an examination of ammonia nitrogen excretion of coho salmon in distilled water at 5°, 10°, 15° and 20°C.
3. body composition analyses of coho salmon during a ten week fast in well water at 12° to 14°C.

A. METHODS AND MATERIALS

1. Determination of Ammonia

Since ammonia is the major end product of protein metabolism in fish (Smith 1929; Wood 1958), one must be able to measure its concentration in ambient waters in order to quantify protein catabolism. The method which is selected must meet certain specifications; these being sensitivity, reproducibility and selectivity. As discussed in the previous section, ammonia toxicity imposes

restrictions on the maximum tolerable concentration of this ion in ambient fish water. Ideally this should be less than 0.1 parts per million ammonia nitrogen. However, at the present level of sophistication of ammonia analysis, concentration measurements in this range are difficult. The added burden for metabolism studies in well water is the level of cations (Table XIV) which renders many methods unusable. Different procedures of ammonia analysis were investigated during the course of this work in the hope of finding one which could be adapted to salmon experiments in well water. Each method attempted proved unfruitful, the technique being unsuitable in one respect or another to present purposes. Many procedures tested were non-sensitive at 0.1 parts per million or could not check the appearance of turbidity. Some were not sufficiently reproducible. Still others introduced possible error due to amino bio-degradation products (methods involving the use of heat or caustic treatments). Methods attempted are listed with their appropriate references in Table XV.

Most procedures in the literature, for the determination of ammonia are based upon one or both of the unique properties of this molecule. The first is that ammonium ion exists in equilibrium with the unionized species (pKeq = 9.22). At physiological (about pH7) and dilute solutions acidic pH's, ammonium ion predominates almost exclusively. The second property is that the gas which is able to diffuse out of solution is released when the medium is rendered highly basic. Often it is desirable to separate ammonia from other substances in solution to prevent interference in subsequent reactions (American Public Health Association et al 1965; Ellender, Armour and Camp 1971; Francirek,

TABLE XIV

A COMPARISON OF THE CATION CONTENT OF TAP WATER, WELL WATER AND SEA WATER

Element	Tap Water* ug/ml (ppm)	Well Water** ug/ml (ppm)	Sea Water*** ug/ml (ppm)
Na	2.3	11.5	11×10^3
K	0.195	4.68	390
Ca	4.0	80	400
Mg	0.96	36	1272
Fe	< 1.12	< 1.12	0.028
Mn	< 1.10	< 1.10	0.006
Cu	< 1.09	< 1.09	0.006
Zn	< 0.975	< 0.975	0.006

*Victoria City water analysed in a Jarrel-Ash (Fisher) Atomic Absorption Spectrophotometer

**Water obtained from a well at this laboratory, analysed on the Jarrel-Ash Spectrophotometer

***Calculated from Spector (1956)

TABLE XV

PROCEDURES ATTEMPTED FOR THE ANALYSIS OF AMMONIA IN WELL WATER

Method	Reference
Indophenol complex	Manabe (1969); Riley (1953)
Fluorescent complex	Sardesai and Provido (1969)
Conway - acid-base titration	Conway and Birne (1933)
- Nesslerization	Ibid.
Nesslerization - steam distillation	Appendix I
Ammonia electrode	Orion Research Incorporated (1971)

Fuchs and Ruzickova 1967; Riley 1953). Advantage is then taken of diffusion, the gas being trapped in acid for subsequent acid-base titration or spectrophotometric analysis.

Due to the inability to find an effectual well water method, it was decided to perform the fish assays for endogenous nitrogen excretion in distilled water. The analysis for ammonia by the Nesslerization technique as outlined by Snell and Snell (1949) was found to be very satisfactory.

In 1856, Nessler first proposed that an alkaline solution of mercuric iodide and potassium iodide could be used as a reagent for the direct determination of ammonia (Nichols and Willits 1934). Since that time there has been a great deal of speculation as to the chemical nature of the reagent and the reaction product (Ciogolea et al 1960; Liebhafsky and Bronk 1948; Nichols and Willits 1934; Vanselow 1940). The latest proposal (Morrison 1971) suggests that Nessler's reagent is a complex of formula K_2HgI_4 which reacts with ammonia in solution to produce the coloured complex $NH_2Hg_2I_3$. The latter product is insoluble in water and exists as a colloid suspension. Whatever the reaction sequence may be, the most important feature of the Nesslerization technique, in the present context is the fact that only ammonium ion is measured (Jacobs 1965). Thus one need not be concerned about interference due to other nitrogenous excretory products of fish. This statement was verified by assays on solutions containing both ammonia and urea.

Several different procedures for the preparation of the Nessler reagent have been reported, all differing in sensitivity and

stability (Nichols and Willits 1934; Snell and Snell 1949; Wirth and Robinson 1933). Investigators found that the use of the reagent was limited since turbidity occurred in solutions of high ionic strength such as sea water. Various methods (American Public Health Association et al 1965; Føyn 1949; Riley 1953; Solórzano 1969; Wattenburg 1931; Wirth and Robinson 1933) were developed to either complex or precipitate the interference, all of which have proved to be unsatisfactory.

The procedure used for the preparation of the reagent in this study was Jackson's modification (Snell and Snell 1949). This solution was easy to prepare and gave consistent results over a period of months. As has been stated previously, Nessler's reagent reacts with ammonia to form a coloured complex which may be analysed spectrophotometrically. Leonard (1963) has reported that the maximum accuracy and working range extends from 350 to 600 nanometres (nm). All determinations in these studies were executed at 410 nm (Snell and Snell 1949) although other workers have reported assays at 436 nm (Moeller 1969) and 428 nm (Apostolache 1962). However a wavelength scan of a Nesslerized solution indicates that absorbance decreases beyond 410 nm.

Following the procedure as given in Appendix I for preparation of samples, standard curves of absorbance versus ammonium ion concentration were plotted. Beer's law was followed over the range 0.05 to 2.0 micrograms per millilitre ammonia nitrogen. The former concentration is the minimum which can be determined with accuracy using a path length of four centimetres. In addition, both within and among discrete preparations of Nessler's reagent, consistent

absorbance values were obtained resulting in an average maximum variation in ammonia nitrogen analysis of 2 per cent.

In addition to the precision of determinations, one should ascertain the stability of the coloured complex. All analyses conducted showed that a Nesslerized solution of ammonium sulfate or of up to 0.02 N hydrochloric acid or of fish water were stable with time (absorbances did not change during a one and one-half hour period at room temperature).

2. "Endogenous" Nitrogen Excretion Measurements in Distilled Water

The procedure involved in this experiment simply consisted of placing a fish in a glass jar of aerated distilled water, temperature equilibrated to 11° to 12°C. and then of withdrawing daily water samples for ammonia analysis by Nesslerization. The fish did not show any external signs of stress in this medium. The young coho salmon selected had been reared from eggs hatched at this laboratory and were of an initial weight range of thirteen to twenty grams. Most often where weight measurements were to be taken of living fish, they were first lightly anaesthetized in a solution of tricaine*. However to avoid any effect of the chemical on the nitrogen excretion of fish, another weighing method was employed. Individual fish were netted, then dropped into a tared container of well water. Thus the weight was obtained by difference. Having been measured, the fish was quickly rinsed with distilled water, then placed in the assay jar. The latter was covered with Saran wrap for protection, the compressed air line passing through a small hole. When a hydrochloric acid trap for air

*(Ethyl m-Aminobenzoate Methanesulfonate) Fraser Medical Supplies Ltd.

ammonia was used, the fish were sealed in an all glass container. The outgoing air was bubbled through a two hundred millilitre acid solution prepared from contant boiling hydrochloric acid.

Daily water samples were withdrawn for analysis by volumetric pipette.

Fasting time and total initial water volumes are compiled in Table XVI (Section B).

Where bacterial numbers were determined, the viable plate count assay was employed (Miles and Misra 1938).

3. Body Composition Analyses during a Ten Week Starvation Period

a) Experimental Animals

Juvenile coho salmon Oncorhynchus kisutch which had been reared from eggs hatched at this laboratory were kept in a tank (three feet in diameter) of continuously running well water (12° to 14°C.) with a maximum water depth of 28.5 centimetres. To obtain initial measurements, individuals were netted and anaesthetized in a tricaine well water solution. Subsequent measurements were performed as stated in "Analytical Methods 3.b)". The fish were then returned to the main tank after a short recovery period in aerated well water. An attempt was made to obtain fish of a uniform size and weight distribution. However due to a limitation in population, a group of fish was selected which ranged in initial weight from approximately twenty to thirty grams. The mean weight of the eighty-five fingerlings selected (Appendix II) was 26.18 grams with a standard deviation of plus or minus 2.63 grams and the mean length was 13.3 centimetres. The

weight distribution was skewed to the left, the median of the sample being 26.79 grams.

b) Analytical Methods

i) Wet Weight and Length

Salmon were netted individually and were then anaesthetized (as a group) in a well water solution of 0.38 mM tricaine. Within a few minutes the fish experienced respiratory collapse and expired. Each one was then drawn out of the water by hand allowing excess water to drain off freely. The fingerling was placed on a fifteen centimetre ruler, the nose tip against a rubber stopper at the zero mark and length was measured from nose to tail fork (fork length). Weight figures were then taken on a Sauter balance to the nearest 0.01 gram. The reproducibility of the weight measurements was determined to be better than 99 per cent.

ii) Dry Weight

Having measured wet weight and length, the fish was cut in two and each piece perforated (one slit through the skin) to enable the escape of water vapour during drying. The samples were subsequently placed in an oven at 85° to 90°C. for twenty-four hours and brought to constant weight.

iii) Fat-free Dry Matter

The dried fish was carefully transferred to a four ounce jar to which was added one hundred millilitres of pesticide grade hexane (Fisher Chemicals 71C #H-300),

having first ensured that the larger fragments were broken up. This mixture was covered and stored in the dark at room temperature for two days, disturbed by occasional swirling. The supernatant was then filtered off through tared Whatman #7 (fifteen centimetre) qualitative fluted filter paper. Another one hundred millilitres of solvent were added, and the solution stored an additional two days. The residue material was then collected on tared filter paper, oven dried at 85° to 90°C. for two and a half to three hours and weighed.

iv) Homogenization of Fat-free Dry Matter (FFDM)

In order to perform subsequent protein and ash content analyses, the FFDM had to be of a homogeneous composition. Therefore, the dried lipid-extracted fish residue was transferred from the filter papers into a Wiley cutting mill. Then the sample was ground using a sixty-mesh sieve, collected and removed to a plastic bag which was sealed. A residue consisting primarily of scale material was frequently found which did not pass through the sieve. This was weighed and stored in glass vials for nitrogen analysis.

v) Micro-Kjeldahl Determinations for Nitrogen

1) Fat-free dry matter (FFDM)

The proteins of animal tissues have traditionally been determined to be 16 per cent by weight of nitrogen. Therefore when quantifying the protein content of an unknown sample, the nitrogen concen-

tration was measured and multiplied by a factor of (100/16). However animal tissues also contain non-protein nitrogenous compounds, the nitrogen content of which if included in the above figure would lead to erroneously high protein levels. Tarr (1958) states that the non-protein nitrogen of teleosts accounts for 9.2 to 18.3 per cent of the total nitrogen. However Bate-Smith (1946) presents a table in which the non-protein nitrogen of fish muscle averages between 0.3 and 1.4 per cent of the total nitrogen, the value for salmon muscle being 0.47 per cent. This latter figure seems applicable to the results of this study. Protein determinations were performed on the fat-free dry material by assaying for nitrogen then multiplying by the (100/16) factor. The protein content in grams was then subtracted from the amount of FFDM to give the quantity of residue or ash. Independent ash content analysis of the FFDM showed that the residue values were too small by 20 per cent. This was manifested as a 0.6 per cent error in the overall body composition analysis. Thus an error of 0.5 per cent in the determination of whole body protein content due to non-protein nitrogen would tally very well.

Nitrogen content was analysed according to the Kjeldahl procedure (Horwitz 1970). The accuracy of each set of analyses was monitored by an internal

ammonium sulfate standard (Fisher Chemicals 71C #A-702, two times recrystallized, dried at 105° to 110°C. for 2.5 to 3 hours) which was treated exactly as were the samples. Titrations were performed using a standard hydrochloric acid solution prepared from constant boiling hydrochloric acid. The average analytical error over a period of ten weeks in the standards assays was 1.8 per cent.

2) Lipid extract

As an internal check for protein losses during body composition analysis, it was necessary to determine whether any nitrogenous compounds were in the hexane washes from the fat-free dry matter. Hexane wash samples were therefore assayed in triplicate against a standard ammonium sulfate solution. Since the hexane solvent was found to interfere in the analytical procedure, it was removed by evaporation either by heat application or in vacuo. The Kjeldahl procedure (Horwitz 1970) for determination of nitrogen was employed, modified only in that ammonia was quantified by Nesslerization (as being more sensitive) rather than by acid-base titration. The amount of nitrogen in the lipid extract material was negligible (0.01 per cent of the total body nitrogen analysed).

vi) Moisture Assay

To ensure that protein determinations were based upon

fat-free dry material, the moisture content of the latter was determined and corrected for. Samples of the homogenized FFDM from their plastic bags were measured into weighing bottles and heated at 85° to 90°C. for three to four hours to constant weight. Moisture content was determined by difference.

vii) Ash Analysis

The determination of the mineral residue content of the fat-free dry material was done according to the procedure in the text edited by Horwitz (1970). The average ash content of the fat-free dry material for weeks of starvation zero to ten was found to be 16.8 plus or minus 0.5 per cent.

B. RESULTS AND DISCUSSION

1. "Endogenous" Ammonia Excretion Assays

In view of the difficulties inherent to the analysis of fish protein metabolism, the following work must be regarded in the nature of a guide to further studies. To ensure accurate quantification of endogenous activity, several criteria must be observed:

a) A nitrogen-free diet should be available to the fish, sufficient to meet metabolic caloric expenditures.

b) Water temperature must remain constant during the assay period.

c) A minimum of environmental stress should be ensured. One must provide for:

i) an adequate (non-limiting) oxygen supply by means of

air-saturated water

ii) the least possible ambient concentration of toxic excretory products such as ammonia

iii) a water environment of ionic concentration adequate to prevent metabolic taxation of regulatory mechanisms.

d) Bacterial growth should be kept to a minimum to prohibit fish disease and the alteration of the nitrogenous compounds which fish excrete, by the multiplication of microflora.

With the fish culture facilities available in this laboratory at the present, protein metabolism studies could be conducted. In the continuous flow well water system employed, the temperature fluctuates between 11° and 14°C. during the year. Thus, although not constant, the variation is low and if monitored during studies would provide adequate reference values. As to the provision of oxygen, the mixing of air with the well water at the tank intakes provides approximately 9.3 milligrams dissolved oxygen per litre. The data compiled by Fry (1971) would indicate that this concentration is certainly sufficient to meet metabolic demands at that temperature range. Oxygen only becomes a limiting factor for the swimming speed of young coho salmon at about eight milligrams per litre (15°C.)(Davis et al 1963).

Ionic stress is a somewhat more nebulous topic. The active or passive excretion of cations in freshwater teleosts has not been thoroughly studied in relation to environmental electrolyte levels. The natural habitat, lakes and streams can vary widely in salt concentration and composition (Hoar 1966). Continual adjustments

maintain serum ion levels within certain limits. Since further data is not available on serum analysis of fingerling salmon, one can not really draw any conclusions as to the adverse or beneficial effects of fish culture in well water.

However, in addition to these previous considerations, the measurement of protein (nitrogen) metabolism poses definite difficulties. First, the provision of a nitrogen-free diet is itself a matter of some concern. Little is known about the ability of fish to digest and metabolize the energy foods - carbohydrates and fats. As a result there is some disagreement as to the maximum tolerable dietary level of carbohydrate (Phillips 1969). Also, considering the low temperature range at which fish grow, fat calories would have to be supplied as unsaturated oils. Body lipid must not be solid if maximum muscular flexibility is to be ensured. However, not only are these oils labile to oxidation but also make a feed mix very difficult to pellet. Finally, assuming that such a ration could be developed, there is no guarantee that the fish would consume it.

The problem of ammonia toxicity must also be broached. The fish tanks in use hold about thirty-three litres of water, one-quarter of which is flushed out every hour. Thus with due attention to the total weight of fish per tank one could attain a limiting ammonia concentration approximating 0.1 micrograms ammonia nitrogen per millilitre. For example:

> Take 10 fish - 10g each; Total 100g fish/tank
>
> If each fish excretes 0.2 mg ammonia nitrogen/g/day, then the total excretion would be about 1 mg/tank/hour.
>
> One-quarter of the total accumulated excretion is removed every hour.

> Therefore when 1 mg ammonia nitrogen represents 0.25 of the total accumulated excretion in one hour, a static state is attained.
>
> This occurs at a concentration of about 0.1 mg ammonia nitrogen/ml.

Although this represents an ideal situation in terms of waste removal, it greatly hampers excreted nitrogen determinations. The primary problem is one of concentration. At 0.1 micrograms ammonia nitrogen per millilitre, not only are many assays nonsensitive but also others become highly susceptible to variable influences. One of the latter is the electrolyte concentration of well water. If the level of ammonia is 0.1 micrograms per millilitre, calcium and magnesium cation concentrations are four to eight hundred times greater. By checking water flow, one could allow excreted ammonia nitrogen to build up to more readily measured levels such as one to two micrograms per millilitre. However, again a distillation method must be employed to eliminate ion interference in colorimetric determinations. In addition, one must realize that by increasing ambient concentrations of ammonia, an aberration in normal elimination may be induced.

In view of these many difficulties, the steps which were taken to obtain data may appear to bear little resemblance to the favourable experimental conditions as discussed above. Nevertheless, the results secured are of value.

There were two basic approaches to the analysis. On the one hand, fish were studied at well water temperatures (11° to 12°C.) and on the other, at a series of four constantly maintained temperatures (5°, 10°, 15°, 20°C.). In both cases, the ammonia excretion of individual fish was assayed in distilled water. Furthermore, all

animals were subjected to a pre-fast period of from twenty-four to seventy-two hours in running well water to ensure minimal faecal contamination during the test (see Table XVI). The actual time of passage of food material from mouth to anus in young salmon has not been measured.

TABLE XVI

CONDITIONS IMPOSED IN AMMONIA EXCRETION ASSAYS

Experiment	Prefast in WW* (Days)	Fast in DW** (Days)	Total Length of Fast (Days)	Volume of DW** (ml)	Water Change After (Day)	Comment
11° - 12°C.						
1	2	5	7	1500	-	HCl trap for NH_3 used
2	3	5	8	1500	-	HCl trap used
3	1	8	9	1500	4	-
5°, 10°, 15°, 20°C.						
1	1	5	6	1500	-	-
2	2	14	16	2000	7	Fish were kept in black lacquer painted jars

*well water

**distilled water

However, Brett and Higgs (1970) studied the rate of gastric digestion at different temperatures. Within 37.8 hours at 9.9°C. and 22.6 hours at 14.9°C., 99 per cent digestion had occurred. Therefore, at the temperature range in these experiments one can assume that within forty-eight hours of fasting faecal excretion has been reduced to a minimum.

The object of the first two trials at 11° to 12°C. was two-fold. First, it was necessary to obtain some estimate of the magnitude of daily ammonia excretion. Second, the loss of ammonia in the air bubbling through the fish jar had to be determined. The fish was therefore sealed in a jar with two outlets - one for input air, the other for air leaving the chamber passing through a hydrochloric acid trap. Upon completion of the assay, the acid was titrated. The results indicated that less than one per cent of the total ammonia excreted was carried away in the water-saturated air stream. Thus ammonia loss by this channel was negligible. Gerking's (1955) analyses corroborate this finding.

An additional system factor examined was the growth of microflora. Bacteria require a source of nitrogen for continued multiplication. This could very well be provided by the ammonia which the fish excretes. Such a situation imposes a potential error in total excreted ammonia measurements. Therefore bacterial numbers were followed for the five day experiments. From an initial population of zero, an increase to one million bacteria per millilitre was observed. The following calculation shows that bacterial growth affects excreted ammonia nitrogen determinations to an insignificant extent.

> If there are 10^6 bacteria/ml after five days, then in 1500 ml, there are about 1.5×10^9 bacteria.
>
> If each cell weighs 10^{-12} g (dry weight) (Brock 1970), then there is a total of 1.5 mg of bacteria.
>
> If dry bacteria are 10% nitrogen (Topley and Wilson 1943), then there is a total of 0.15 mg nitrogen.
>
> Therefore 0.15 mg ammonium nitrogen would be used in protein synthesis.

> If a total of 16 mg ammonia nitrogen was excreted over the same time period by a 10g fish then a 0.9% error was incurred in the nitrogen analysis by bacterial growth.

Several assumptions guided these calculations, the prime one being that the bacteria were actually multiplying. They may have been gradually released from the mucoid skin surface of the fish or from the digestive tract.

The above results are corroborated by other workers who have reported that over a twenty-four hour period in both fresh and sea water, bacterial action on waste products has been negligible (Fromm 1963; Gerking 1955; Wood 1958).

In Table XVII the results of the three 11° to 12°C. experiments are compiled. Examination of the data leads to a few tentative conclusions. Although ambiguous results may be expected as a result of large ambient ammonia concentrations, there does appear to be a significant difference between the excretion rates in assays one and two compared to three. The mean five day excretion rate for the former groups averages about 0.21 milligrams ammonia nitrogen per gram per day. For a comparable length of total fast (seven days in distilled water) in group three, the rate averages 0.14 milligrams ammonia nitrogen per gram per day. This difference could be due to several factors. One of which is that ammonia excretion may be inhibited due to a longer fast in distilled water. Or, groups one and two may be excreting at a higher rate due to stress from the small positive pressure in the system (acid traps in series). On the other hand, these fish may have been excited due to additional handling (one extra transfer from well water to distilled water).

TABLE XVII

AMMONIA NITROGEN EXCRETION OF YOUNG SALMON
(11° - 12°C.)

Results are given in mg ammonia nitrogen/g fish/day

Length of Fast in DW* (Days)	Experiments 1 Fish 18.24g**	1 Fish 14.4g	1 Fish 15.85g	1 Fish 20.0g	2 Fish 12.8g	2 Fish 11.7g	3 Fish 13.3g	3 Fish 14.6g
1	.238	.212	.206	.352	.228	.233	.162	.207
2	.230	.183	.220	.233	.110	.150	.144	.151
3	.262	.188	.230	.255	.143	.156	.093	.121
4	.203	.209	.196	.265	.196	.243	.135	.125
5	.175	.202	.177	.260	.222	.226	.146	.140
mean of five days	.222	.199	.206	.273	.180	.202	.136	.149
6							.081	.063
7							.210	.185
mean of seven days							.139	.141
8							.083	.043
mean of eight days							.132	.129

*distilled water

**initial wet weight of fish

The selection of means for purposes of comparison may be justified on the grounds that a consistent pattern of nitrogen excretion did not emerge. Therefore the mean more closely represents all the data.

One way of placing these ammonia nitrogen excretion results in perspective is to make an estimate of the level of nitrogen elimination

when the fish is subjected to non-fasting conditions. Feed intake and body weight measurements indicate that when a fish is fed a diet containing 60 per cent protein, it consumes one per cent of its body weight per day. Thus a one hundred gram fish would eat one gram of feed per day equivalent to 0.6 gram protein or 0.096 gram nitrogen. If none of this nitrogen were retained in the body, the fish would excrete 0.96 milligrams nitrogen per gram fish per day. This theoretical rate of excretion is approximately five times that observed in these fasting experiments.

The seond approach to this study was the examination of ammonia nitrogen excretion as a function of temperature. Since the metabolic rate of a poikilotherm depends upon temperature, one would expect a more rapid turnover of body protein at elevated temperatures and thus an increase in rate of ammonia excretion (Bonnet 1933). These experiments were conducted in a manner similar to the previous assays (Table XVI). However the temperature of the fish jars was maintained within a range of plus or minus 0.5°C. by a four compartment water bath system of cold water coils and individual heaters. The results for these ammonia assays are presented in Table XVIII.

Perusal of the data indicates certain discrepancies. For example, in experiment one, definite differences are apparent for rate of excretion, the highest rate being at 15°C. However, in experiment two, the rate of ammonia excretion is highest at 20°C., decreasing in order of 15°, 10° and 5°C. Excretion at 10°C. in both experiments at the five day mean (0.140, 0.138 mg ammonia nitrogen/g/day) is very comparable to the rate obtained in the 11° to 12°C. assay - experiment three (0.136, 0.149 mg ammonia nitrogen/g/day).

TABLE XVIII

AMMONIA NITROGEN EXCRETION OF YOUNG SALMON AT FOUR TEMPERATURES

Results are given in mg ammonia nitrogen/g fish/day

Length of Fast in DW* (Days)	Experiments 1 20°C. 14.0g**	15°C. 15.3g	10°C. 15.0g	5°C. 15.4g	2 20°C. 16.7g	15°C. 17.3g	10°C. 15.6g	5°C. 17.8g
1	.444	.446	.206	.142	.353	.266	.137	.128
2	.214	.273	.111	.090	.211	.186	.249	.067
3	.178	.327	.175	.096	.299	.207	.038	.026
4	.293	.327	.133	.117	.240	.254	.148	.057
mean of four days	.282	.343	.156	.111	.276	.228	.143	.070
5	.243	.360	.075	.102	.078	.202	.121	.039
mean of five days	.274	.347	.140	.109	.236	.223	.138	.063
6					.270	.347	.237	.124
7					.180	.098	.231	.039
mean of seven days					.233	.223	.166	.070
8						.282	.247	.067
9						.104	.115	.067
10						.143	.194	.108
11						.183	.084	.067
12						.226	.173	.093
13						.006	.321	.062
14						.168	.115	.064
mean of fourteen days						.191	.172	.072

*distilled water
**initial wet weight of fish

The reliability of this set of results must be tempered with a degree of caution. This is necessitated by three factors adjunct to those previously discussed. First, there is a question of a sudden change in hydrogen ion concentration. The pH of well water is close to eight. That of the distilled water is initially about 5.6. The transfer from one medium to another must then involve adjustments in the diffusion from the blood through the gills of substances (such as bicarbonate and possibly ammonium ions) dependent upon a pH gradient. Second, a transfer from 11° to 12°C. water to 20°, 15°, 10° and 5°C. water probably results in a thermal shock. Effects of temperature changes in fish have not been thoroughly studied. However, workers have reported that various salmonids are able to adjust at a rate of 1°C. per hour (Fry 1971). If such is the case then well within the first twenty-four hour period in distilled water, the metabolic rates of all the fish assayed were temperature acclimated. This is a rather rapid adaptation. It is therefore not surprising that only one fish, the first studied at 20°C. in experiment two died within thirty-two hours after transfer. Finally one must consider the effect of excitement which is a poorly defined parameter in fish studies. As earlier emphasized, the imposition of conditions suitable for basal metabolic studies restricts animal activity to a minimum. While the effect of stimuli such as temperature have been assessed, the impression of others (for example, light, water turbulence, method of aeration, rapid movements in the environment, handling, crowding, anaesthetization) on the metabolic rate of a fish is unknown.

Be that as it may, one has to begin the study of a new system at some point. Thus the conclusions to be drawn from the present data

are subject to the revision of future work.

Since the data of experiment two more closely correspond to the expected pattern of rise in metabolic activity with temperature, calculations will be based upon these results. One of the first noticeable features is the pattern of nitrogen excretion at the four temperatures. The van't Hoff rule states that within certain limits for each increase of 10°C. in temperature, the speed of chemical reactions is doubled (Brody 1964; Gortner and Gortner 1949). This relationship is often expressed in biological systems as the quantity Q_{10} which is the ratio of the rates of reaction at two temperatures ten degrees apart. For most enzyme systems at lower temperatures (zero to 10°C.), the Q_{10} is about two, gradually diminishing with temperature increase (Gortner and Gortner 1949). If the ratios of milligrams of excreted ammonia nitrogen per gram of fish wet weight per day are calculated one obtains the following Q_{10} values:

Q_{10}		Ratios
3.54	=	$\frac{0.223_{15°C.}}{0.063_{5°C.}}$
1.71	=	$\frac{0.236_{20°C.}}{0.138_{10°C.}}$

These results correspond to the predicted pattern.

The computation of the rate of "endogenous level" nitrogen excretion (as a product of protein metabolism) is based upon the five day means of ammonia nitrogen excretion. There are a number of reasons for this choice. First, a balance had to be chosen. On the one hand, there may have been an initial decrease in rate of excretion due to the degradation of labile protein stores. On the other, there may have been

a rise in rate of ammonia excretion due to prolonged fasting. Second, it was desirable to avoid the high levels of ambient toxic ammonia that develop with time in unchanged water. Third, a mean value eliminated concern over untoward daily variability. Therefore the five day mean (which corresponded to a total fast of seven days length) was chosen as the more representative figure for rate of nitrogen excretion.

Using this result, one can proceed to calculate the ratio of "endogenous" nitrogen excretion to standard heat production (Table XIX). This figure is valuable in terms of comparative nutrition. In other words, the relationship of certain fish and mammalian biochemical parameters provides a measure of the homogeneity of biological systems. At 10°C. for the fish in this experiment, the ratio is thirty-one milligrams excreted nitrogen per kilocalorie as compared to two milligrams per kilocalorie for the mammal. Therefore one can conclude that the fish may catabolize protein to a much greater extent. Or, more probably the nitrogen excretion figures here obtained bear no relationship to endogenous values and/or standard metabolic rate data is invalid or not applicable to the situation at hand.

As a comparative measure, one can examine the findings of other workers in the field. Using the nitrogen excretion data of Fromm (1963) for a fasting rainbow trout and an approximate figure for standard metabolic rate from the data of Beamish (1964b) and Brett (1971) a similar ratio may be calculated. The rainbow trout kept at 12° to 14°C. had after six days fasting, an ammonia nitrogen excretion rate averaging eighty milligrams nitrogen per kilogram per day. One can estimate the standard oxygen consumption to be about sixty milligrams per kilogram per hour. Therefore the ratio of rate of endogenous

TABLE XIX

RELATIONSHIP BETWEEN NITROGEN EXCRETION AND ENERGY METABOLISM FOR SALMON IN EXPERIMENT TWO (TABLE XVIII)

Temperature °C.	A Rate of Ammonia Nitrogen Excretion* (mg/kg fish/day)	B Standard Metabolic Rate** (kcal/kg fish/day)	Ratio of A to B (mg ammonia nitrogen/ kcal)
5	63	3.65	17.3
10	138	4.46	31.0
15	223	5.26	42.4
20	236	6.05	39.0

*data from Table XVIII

**data from Brett (1971) (Table VIII)

ammonia nitrogen excretion to standard metabolic rate is 16.5 milligrams ammonia nitrogen per kilocalorie, half as large as the ratio calculated in this study. However, both of the above results appear to bear little relationship to the ratio 9.34 milligrams excreted nitrogen per kilocalorie which Benedict obtained for the homeothermic rat after a ten day fast.

The fact that nitrogen excretion figures are probably grossly exaggerated (due to a multitude of stimuli) has been previously discussed. On the other hand, a few more words are required on the matter of standard metabolic rate. To be truly valuable both standard metabolic rate and endogenous nitrogen excretion should be expressed as functions of body weight. In mammalian and some fish (Beamish 1964a; Wohlschlag and Juliano 1959) studies workers have shown that metabolic

activity at one body weight does not necessarily correspond to that at another. In other words, extrapolation of the nitrogen excretion rate from a kilogram of fish to a ten gram fish merely by dividing by a factor of one hundred is not necessarily valid. The same criteria may apply to standard metabolic rate. Therefore when determining ratios as in Table XIX one may be trying to compare at each individual temperature, fish of completely different weight ranges. This inconsistency may account for some part of the error realized. Even so, the most probable explanation is that nitrogen excretion data was not endogenous nor the metabolic rate of the assay fish at a standard level.

2. Body Composition Study of Fasting Oncorhynchus kisutch

Body composition studies are highly useful as a means of assessing the effect of environmental variables upon tissues. In this context, the term environment means the absence of a food supply. Not only does fasting data provide insight as to body component inter-relations but also can be used to estimate body nitrogen expenditure.

With these thoughts in mind, one can proceed to examine the results of a ten week starvation experiment using coho salmon. Following the withdrawal of an initial sample, fish were killed at weekly intervals and analysed for water, fat and protein content. These raw data are presented in Appendix III. The relative magnitude of the particular body component was then determined as a percentage of the wet body weight for each fish and averages were taken as representative of the composition at each sample week. The result of these calculations is presented in Table XX. Relative (percentage)

rather than absolute (grams) composition figures are more meaningful in this experiment since the initial weights of the total sample were not normally distributed, nor the weekly sampling necessarily uniform.

Upon analysis of Table XX, it is immediately apparent that definite trends in body composition developed with duration of starvation. In accordance with tendencies reported in mammalian and other fish studies, fasting results in a percentage increase of body water and a decrease of protein and fat. Lipid reserves are rapidly depleted and by week five probably only the essential lipid compounds remain intact. The body protein content, on the other hand remains relatively constant up to week four after which a gradual decrease is observed. Obviously, energy metabolism draws upon fat until the bulk of reserves is depleted after which protein is mobilized. In comparison, the percentage water content increases gradually over the entire course of starvation while the residue (ash) remains constant within the error limits of the determinations. The analysis of ash content by an independent method (Methods) and subsequent comparison to the per cent residue of Table XX provided an internal accuracy check. For starvation weeks zero to ten, the ash content of the fat-free dry matter remained constant, the mean value 16.8 plus or minus 0.5 per cent. This uniformity is probably indicative of an error in the determination of ash. The average ash content of the fat-free dry fish as determined above was larger by 20 per cent than the residue value. Nevertheless, this amounted to an analytical error of only 0.6 per cent on a wet body weight basis.

While general composition changes yield a certain amount of information, the analysis of protein metabolism requires a somewhat more

TABLE XX

BODY COMPOSITION OF FASTING SALMON

AT 12° TO 14°C.

Length of Fast (weeks)	Per Cent of Body Wet Weight (%)			
	Water*	Protein*	Lipid*	Residue**
0***	74.6	17.1	5.8	2.5
1	75.5	17.1	5.0	2.4
2	75.3	16.5	5.7	2.6
3	76.9	16.9	3.6	2.6
4	76.8	16.8	3.8	2.6
5	78.7	16.4	2.1	2.7
6	78.4	16.9	1.7	3.0
7	79.5	15.5	2.3	2.7
8	80.2	15.5	1.5	2.6
9	80.9	15.1	1.0	2.9
10	82.0	14.1	1.2	2.7

*Each result is an average of seven samples for weeks of fast one to ten.

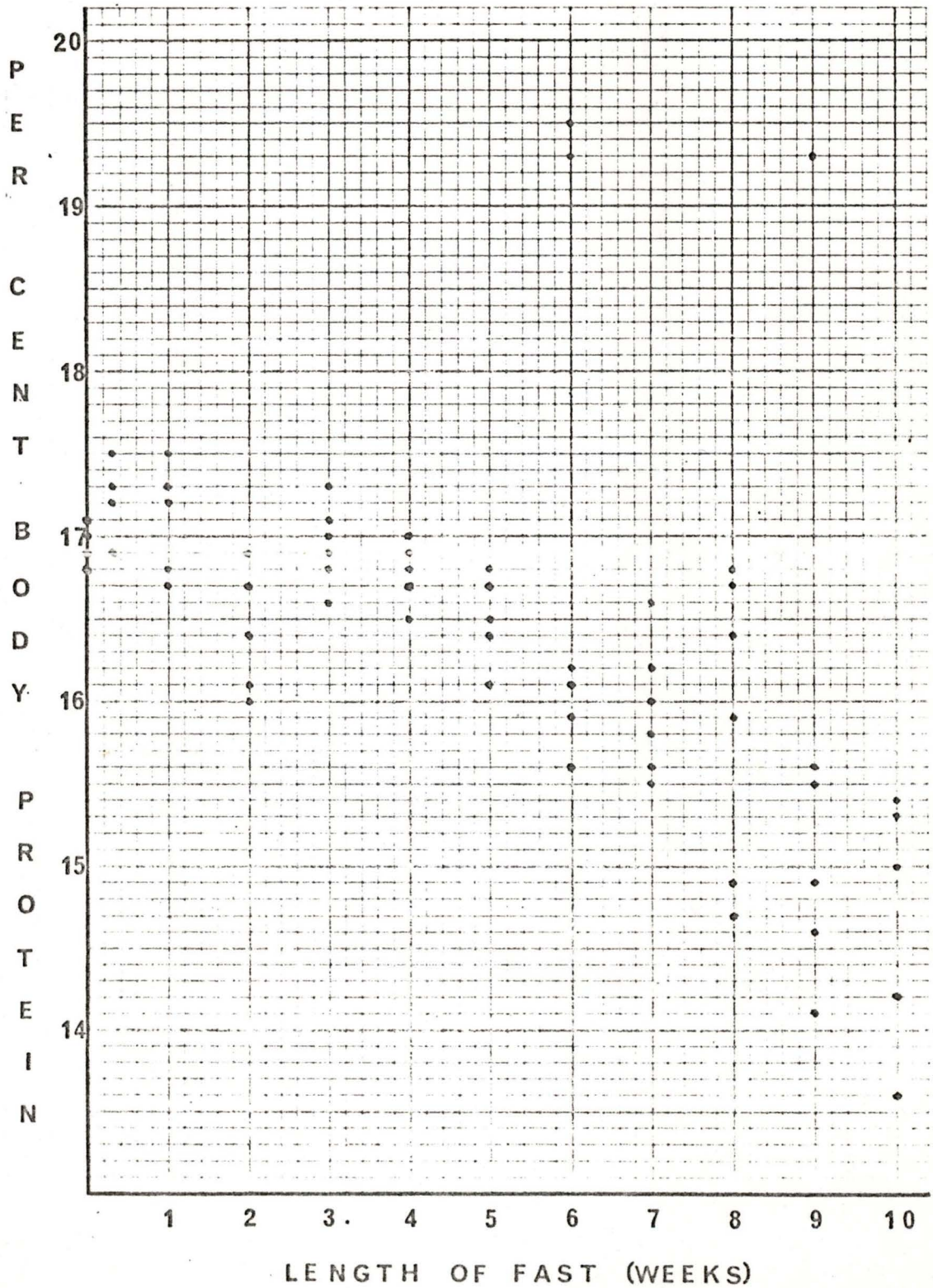
**Residue g = Fat-free dry matter g - (Protein g + Lipid g)

***The zero week sample figures are averages for five non-fasted fish and five two day fasted fish.

quantitative interpretation. To this end, an attempt was made to fit a regression equation to the data. Examination of the scatter diagram (Figure 1) for the plot of per cent protein of each fish analysed versus week of fast indicated that a simple linear relationship could not be assumed. However the data did appear visually to correspond to a curvilinear function. Therefore by the method of least squares outlined

FIGURE 1

PER CENT WET BODY WEIGHT PROTEIN VERSUS LENGTH OF STARVATION FOR INDIVIDUAL SAMPLES OF COHO SALMON



by Croxton and Cowden (1939) and with the aid of a computer programme for curvilinear regression, a second degree polynomial equation was determined of the following form:

$$y = 16.78625 + 0.1868356x - 0.03739294x^2 \text{ or}$$
$$y = 16.8 + 0.187x - 0.0374x^2$$

y = per cent protein of wet body weight

x = length of starvation in weeks + 1

(For the above curve fitting, the initial and two day fast body composition data were combined to represent the unfasted sample.) The standard error of estimate and the coefficient of curvilinear correlation were computed to be 0.813 per cent and 0.753 respectively. Although the correlation coefficient is significant at the 0.01 level of significance (Richmond 1964), the value indicates a relatively poor approximation of the above equation to the data. However, this is not a surprising outcome. It seems unlikely that the body protein content should be highly correlated to the length of starvation. Too many other factors play a crucial role, body fat reserves and their oxidation and change in metabolic rate being of prime importance. The large standard error of estimate is explained by the wide spread in data.

In spite of the obvious imperfections of the regression equation, one can still derive certain valuable information with its use. For instance, one can estimate the daily rate of protein oxidation and hence nitrogen excretion over the ten week period (Table XXI). As is clear from the results, both activities are undetectable until three weeks starvation, after which rate of catabolism increases. Endogenous nitrogen excretion as inferred from this Table is therefore less than twenty-three milligrams per kilogram fish per day as compared

TABLE XXI

RATE OF PROTEIN OXIDATION AND NITROGEN EXCRETION

IN THE FASTING SALMON

Length of Fast (weeks)	Wet Body Weight Protein* (%)	Protein Oxidized (%/week)	Protein Oxidized (mg/kg fish/day)	Energy Release** (kcal/kg fish/day)	Nitrogen Excretion*** (mg/kg fish/day)
1	17.0				
2	17.0				
3	16.9	0.1	140	0.66	23
4	16.8	0.1	140	0.66	23
5	16.6	0.2	290	1.4	46
6	16.3	0.3	430	2.0	69
7	15.9	0.4	570	2.7	91
8	15.4	0.5	710	3.3	110
9	14.9	0.5	710	3.3	110
10	14.3	0.6	860	4.0	140

*per cent body protein calculated from the second degree polynomial regression equation

**caloric equivalent of protein taken to be 4.7 kilocalories per gram

***protein is assumed to be 16 per cent by weight nitrogen, all of which is excreted upon catabolism

to about two hundred milligrams nitrogen excretion per kilogram per day determined in the previous distilled water experiments. This power of ten difference would then bring the ratio of milligrams endogenous nitrogen excretion to basal kilocalories within the range of values reported for mammalian analyses.

For example, let us assume that the endogenous nitrogen excretion of a salmon is about 20 milligrams nitrogen per kilogram per

day at 10°C. From Brett's data (Table VIII), at 10°C. the standard metabolic rate of the sockeye salmon is 4.46 kilocalories per kilogram per day. The ratio of the rate of endogenous nitrogen excretion to the standard metabolic rate would then be 4.5 milligrams nitrogen per kilocalorie.

Since there was some doubt as to whether a second degree or third degree polynomial regression equation was a better expression of the data in this experiment, a test was performed for goodness of fit. This indicated that there was a significant quadratic trend (P less than 0.001). Therefore the second degree polynomial equation was used in the fore-going analysis of protein catabolism during starvation. Primarily for reference purposes, the third degree polynomial expression (as determined by computer programme) and related analysis are included in Appendix IV.

To obtain a more objective representation of fat catabolism with length of fast, a regression equation was calculated (by the least squares method of Croxton and Cowden (1939)). The result was a second degree polynomial equation of the following form:

$$y = 6.1781 - 0.858229x + 0.0339789x^2 \text{ or}$$
$$= 6.18 - 0.858x + 0.0340x^2$$

y = per cent fat of wet body weight

x = length of starvation in weeks

The standard error of estimate and the curvilinear correlation coefficient were computed to be 1.26 per cent and 0.804 respectively. The latter was significant at the 0.01 level. The addition of a third degree term was not deemed significant. Using the above expression, one can calculate the per cent body fat versus time of fast and thus determine the rate of heat production (Table XXII). It appears that

TABLE XXII

RATE OF LIPID OXIDATION AND CONCOMITANT ENERGY PRODUCTION

Length of Fast (weeks)	Wet Body Weight Lipid* (%)	Lipid Oxidized (%/week)	Lipid Oxidized (g/kg fish /day)	Energy Release by Lipid Oxidation** (kcal/kg fish/day)
0	6.18			
1	5.35	0.83	1.2	11.4
2	4.60	0.75	1.1	10.4
3	3.91	0.69	1.0	9.5
4	3.29	0.62	0.9	8.6
5	2.74	0.55	0.8	7.6
6	2.25	0.49	0.7	6.6
7	1.84	0.41	0.6	5.7
8	1.49	0.35	0.5	4.8
9	1.21	0.28	0.4	3.8
10	0.99	0.22	0.3	2.8

*per cent body lipid calculated from second degree polynomial regression equation

**caloric equivalent of fat is taken to be 9.5 kilocalories per gram.

fat is catabolized at a constantly decreasing rate (minus 0.01 per cent per day). Subsequently the heat production due to fat oxidation diminishes at a rate of about one kilocalorie per kilogram fish per day.

Moreover, based on the assumption that the oxidation of fat and protein solely accounts for total energy metabolism, then, during the first week of starvation (by Tables XXI and XXII) the metabolic rate of the fish was 11.4 kilocalories per kilogram per day. In

comparison, if the fish in this experiment were swimming at one fish length per second, then from Brett's (1971) data, there would be an oxygen consumption of 120 milligrams per kilogram per hour. This corresponds to an energy metabolism of 9.7 kilocalories per kilogram per day. The latter differs by 17 per cent from the experimentally determined value. This discrepancy is not unreasonable considering the nature of the assumptions involved in these calculations.

Most importantly, the regression equation for fat catabolism enables one to relate protein and energy metabolism in the fasting fish. To this end certain approximations must be made as to the caloric equivalent values for protein and fat. The heat of combustion of proteins and fats in meats has been found to be 5.65 and 9.5 kilocalories per gram respectively (Crampton and Lloyd 1959). Since the fasting animal is metabolizing its own tissue energy compounds, no loss of the gross energy of fat or protein occurs due to waste in the digestive tract. Therefore the metabolizable (or physiological fuel) value of these compounds differs from the gross available energy only in the energy loss due to the excretion of nitrogenous by-products. If urea constitutes the bulk of nitrogenous excreta, one can calculate the metabolizable energy of protein to be 4.8 kilocalories per gram. On the other hand, if ammonia excretion is of major significance (as in fish), the metabolizable energy of protein is 4.7 kilocalories per gram. Therefore, in this study, when the heat production due to protein and fat catabolism is determined, the caloric equivalent values are taken to be 4.7 and 9.5 kilocalories per gram respectively.

Since the weight loss of body fat and protein can be related to a corresponding heat production, the ratio of the rate of endogenous

nitrogen excretion to metabolic rate can be estimated. Table XXIII presents the results of this calculation.

TABLE XXIII

CONTRIBUTION OF PROTEIN OXIDATION TO TOTAL ENERGY METABOLISM IN THE FASTING SALMON AND RATIO OF THE RATE OF NITROGEN EXCRETION TO METABOLIC RATE

Length of Fast (weeks)	Total Energy Expenditure* (kcal/kg fish /day)	Contribution of Protein Oxidation to Total Energy Metabolism (%)	Ratio** (mg excreted nitrogen/kcal)
1	11.4	-	-
2	10.4	-	-
3	10.2	6.5	2.3
4	9.3	7.1	2.5
5	9.0	16	5.1
6	8.6	23	8.0
7	8.4	32	11
8	8.1	41	14
9	7.1	46	15
10	6.8	59	21

*summation of energy release by oxidation of lipids and protein, Tables XXI and XXII respectively

**ratio of rate of nitrogen excretion (Table XXI) to rate of total energy expenditure

Analysis shows (as did Benedict's data for the fasting rat (Table IV)) that total energy metabolism (combined protein and fat catabolism) decreases with duration of fast. However, protein catabolism becomes increasingly important to heat production. Consequently, the level of

nitrogen excretion increases as does the ratio of nitrogen excreted to energy metabolism. At three weeks of starvation, the above ratio is 2.3 milligrams nitrogen per kilocalorie, closely comparable to the traditionally accepted ratio of 2 milligrams endogenous urinary nitrogen per basal kilocalorie for mammals.

The relationship of protein and fat catabolism during this fast can be examined in another way. In Table XX, it was apparent that about week five, marked changes occurred in the depletion curves of body fat and protein. Lipid stores diminished rapidly to this point after which a more gradual decrease was observed. Protein was mobilized to a limited extent up to week five after which it was more markedly catabolized. Although they are crude approximations, the calculations of Table XXIV clearly show the subordinate position of protein oxidation to fat when the latter is available. Interestingly, the energy production by oxidation of both fat and protein between zero and five weeks of starvation (34.6 kcal/100g fish) is 25 per cent of the total energy reserve (138.6 kcal/100g fish). After this point protein oxidation is more significant, corresponding to the observations of Pentov, Mentov and Kurnaev (1928) in the migrating salmon.

For purposes of comparison of these starvation body composition results to the work of others, one can examine Phillip's data for brook trout (Table X). Unfortunately his sampling technique resulted in an erratic pattern of observed changes in per cent fat and protein. Thus only general trends are clear except for his protein, fat figures at 14.4°C. With the latter, after six weeks of starvation, a more rapid decrease in the percentage of both components appears to occur. Over ten weeks at 14.4°C. protein oxidation accounts

TABLE XXIV

PER CENT OF TOTAL ENERGY METABOLISM DUE TO

PROTEIN OXIDATION IN THE FASTING SALMON

Parameters		Initial Values	Changes during Fasting Period* (weeks) 0 - 5	6 - 10	0 - 10
Body Component (g/100g)	Fat	6.18	3.44	1.75	5.19
	Protein	17.0	0.4	2.3	2.7
Energy Release Represented by Oxidation of Above** (kcal /100g)	Fat	58.7	32.7	16.6	49.3
	Protein	79.9	1.88	10.8	12.7
Total Energy Release (kcal/100g)		138.6	34.6	27.4	62.0
Ratio of Energy Release by Protein Oxidation to Total Energy Production (%)			5.4	39	20

*as determined in Tables XXI and XXII

**caloric equivalents of 9.5 kcal/g and 4.7 kcal/g are assumed for fat and protein respectively

for 33 per cent of the total energy release. Compare this to the ratio obtained in this study of 20 per cent. Clearly one must be prepared to encounter variability both between species and between different studies of this kind.

Further comparison of these two sets of data shows a per cent water content increase in both, with fasting. Is this merely a relative increase or is the absolute body water content actually augmented. Analysis of the raw weights data (Appendix III) for salmon,

indicates that for two fish of comparable body weights, the animal which has been fasted for ten weeks contains more water than the non-fasted one. However the fasted animal has lost dry body substance. The body water of an initially larger fish could be merely retained.

One major discrepancy between the brook trout and salmon data is the change in per cent ash content in the former. However this is explained by the increase in relative water in the salmon of 7 per cent compared to 2 per cent in the trout. In the salmon, the per cent protein and lipid decrease is compensated for by the water level increase rather than a relative rise in per cent ash.

The determination of protein in both of these body composition analyses is subject to criticism. This component is normally determined by nitrogen analysis and use of the 100/16 conversion factor. The above method poses a potential threat to accuracy by way of the non-protein nitrogen fraction. Protein quantities may be over-estimated. Thus, the decrease in fish body nitrogen content may in some part be due to excretion of low molecular weight nitrogenous compounds rather than to protein catabolism. While the former are believed to constitute but a small percentage of total body nitrogen, until reliable analyses have been published, one must be prepared to have the results of this study challenged.

At some point in the discussion of salmon protein metabolism studies, one realizes that there is an inconsistency between the expected pattern of results and actual values obtained. The problem in brief is the discrepancy between the nitrogen excretion of the starved salmon in well water and of those in distilled water. The fasted fish in the ten week experiment exhibited a very low level of nitrogen

excretion for four weeks. Presumably this value is close to endogenous, the mobilization of body fat stores supplying metabolic energy. Now having observed that an exogenous or endogenous energy food will spare protein oxidation, the high rate of nitrogen excretion (implying protein oxidation) of the starved fish in distilled water is puzzling. The fact that the fish were in distilled water is not really a relevant point since large rates of excretion have been reported by others in a more electrolyte-balanced medium (Fromm 1963). If a high metabolic rate is involved why is not body fat used as the alternative fuel during the initial period of fasting? The degradation of labile protein stores would lead to increased nitrogen excretion initially. However the rates observed continued over two weeks of starvation. If 0.2 milligrams nitrogen per gram of fish per day were excreted, then in one week a twenty gram fish would oxidize 0.18 gram protein. The starving twenty gram fish in well water lost about one-half as much protein in five weeks. Therefore, one cannot assume that the oxidation of labile protein is a significant factor. Perhaps a more probable explanation is that the fish in the distilled water assays were excited due to handling and space restriction in the jar as well as to the daily water sampling. The catabolism of the tissue amino acid pool may have been a more rapid immediate source of energy. Consequently new protein could not be synthesized and an overall protein oxidation would result. The fish in well water would be relatively undisturbed and could adjust more efficiently to the imposition of starvation.

In conclusion, the results of this study appear to indicate that the assay for endogenous nitrogen excretion (as ammonia nitrogen)

of individual fasting coho salmon in distilled water at temperatures of 5°, 10°, 11° to 12°, 15° and 20°C. leads to exaggerated nitrogen elimination. The determination of nitrogen loss by body composition analysis of fasting salmon yielded a tentative value for endogenous excretion of 23 milligrams nitrogen per kilogram fish per day. Moreover, the ratio of rate of endogenous nitrogen excretion to metabolic rate was calculated to be 2.3 milligrams excreted nitrogen per kilocalorie.

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APPENDIX I

METHODS OF AMMONIA ANALYSIS

a) Nesslerization

water sample (25 ml)

↓

30 ml test tube

↓ 1 ml Nessler's reagent

1) cap with parafilm

↓

2) invert tube six times

↓ 10 minutes at room temperature

3) read absorbance at 410 nm

b) Nesslerization - steam distillation

micro-Kjeldahl distillation apparatus

↓ steam through 15 minutes

1 ml 2.7 N HCl

sample addition (50 ml)

↓ condenser tip into 0.10 N HCl trap (5 ml) in 25 ml volumetric flask

heat until reflux line is in top chamber

↓ 1 ml 10 N KOH

collect 20 ml distillate

↓ make up to mark

remove sample volume (15 ml)

↓ 0.5 ml Nessler's reagent

proceed as above 1, 2, 3

APPENDIX II

INITIAL WEIGHTS AND LENGTHS OF SALMON USED IN THE FASTING BODY COMPOSITION EXPERIMENT

Fish Weight (g)	Fish Length (cm)	Fish Weight (g)	Fish Length (cm)	Fish Weight (g)	Fish Length (cm)
24.53	13.4	25.68	13.3	29.79	14.0
27.92	13.5	23.32	13.3	25.79	13.1
27.53	13.5	26.79	13.4	27.29	13.6
24.29	12.9	27.55	13.7	29.12	13.9
27.40	12.8	28.89	13.3	27.74	13.7
26.67	13.4	28.29	13.6	26.17	13.9
22.48	12.9	26.84	13.4	29.35	13.6
27.67	13.7	24.02	13.2	27.70	13.8
28.63	13.6	27.56	13.4	20.42	12.4
25.35	13.1	25.88	13.2	21.57	12.5
27.09	13.5	27.39	13.7	21.11	12.6
27.91	13.8	27.27	13.1	22.38	13.1
25.75	13.3	23.43	12.8	21.07	12.8
26.16	13.4	28.35	13.3	29.42	13.8
22.89	13.0	28.94	13.8	24.97	13.4
26.26	12.9	29.44	13.7	20.26	12.3
25.17	13.2	26.60	13.6	22.13	12.8
29.67	14.0	23.99	13.3	29.80	13.8
28.54	13.7	29.14	13.9	28.29	13.4
24.30	13.2	28.34	14.0	22.21	12.6
25.60	13.1	29.68	13.7	20.84	12.4
24.08	12.9	29.40	13.8	23.15	12.4
26.18	13.2	29.56	14.0	21.36	12.5
28.53	13.9	28.62	13.9	29.15	13.8
27.07	13.7	27.74	13.9	20.63	12.6
27.00	13.9	23.93	13.0	28.90	13.5
27.50	13.6	27.29	13.3	24.64	13.1
25.33	13.2	26.29	13.2		
26.53	13.2	26.24	13.9		

APPENDIX III

BODY COMPOSITION OF SALMON SAMPLED DURING A TEN WEEK FAST

Length of Fast (weeks)	Fish Sample Number	Wet Weight (g)	Water Content (g)	Protein Content (g)	Lipid Content (g)	Residue* (g)
0	1	27.97	20.07	4.73	2.47	0.70
	2	19.47	14.73	3.31	0.94	0.49
	3	22.94	17.25	3.91	1.20	0.58
	4	24.87	18.45	4.25	1.54	0.63
	5	22.76	17.18	3.82	1.19	0.57
	mean	23.60	17.54	4.00	1.47	0.59
(2 days)	1	28.66	21.57	4.97	1.39	0.73
	2	29.62	21.68	5.18	2.00	0.76
	3	28.37	21.19	4.88	1.58	0.72
	4	23.13	17.47	3.97	1.10	0.59
	5	24.36	18.39	4.12	1.24	0.61
	mean	26.83	20.06	4.62	1.46	0.68
1	1	28.03	21.04	4.84	1.47	0.68
	2	21.83	17.01	3.75	0.54	0.53
	3	21.77	16.24	3.63	1.39	0.51
	4	19.82	14.63	3.33	1.39	0.47
	5	20.81	15.86	3.64	0.80	0.51
	mean	22.51	16.96	3.84	1.07	0.54
2	1	26.56	19.95	4.44	1.49	0.68
	2	26.91	20.12	4.40	1.71	0.68
	3	20.55	15.31	3.28	1.45	0.51
	4	19.53	14.84	3.14	1.06	0.49
	5	23.84	17.73	3.99	1.51	0.61
	6	24.99	18.99	4.23	1.12	0.65
	7	21.19	16.13	3.54	0.97	0.55
	mean	23.37	17.59	3.86	1.33	0.60
3	1	25.02	18.91	4.33	1.11	0.67
	2	25.95	19.81	4.43	1.03	0.68
	3	24.40	18.40	4.05	1.33	0.62
	4	26.15	20.55	4.39	0.53	0.68
	5	23.37	18.24	3.94	0.58	0.61
	6	25.07	19.62	4.23	0.57	0.65
	7	26.90	20.43	4.56	1.21	0.70
	mean	25.26	19.42	4.28	0.90	0.66
4	1	26.29	20.25	4.43	0.93	0.68
	2	21.65	16.25	3.57	1.28	0.55

*residue = dry matter g - (protein g + lipid g)

Length of Fast (weeks)	Fish Sample Number	Wet Weight (g)	Water Content (g)	Protein Content (g)	Lipid Content (g)	Residue* (g)
	3	21.09	16.31	3.52	0.71	0.55
	4	24.22	18.75	4.06	0.80	0.62
	5	24.43	19.05	4.16	0.58	0.64
	6	24.47	18.79	4.16	0.88	0.64
	7	26.45	20.10	4.45	1.21	0.69
	mean	24.08	18.50	4.05	0.91	0.62
5	1	24.07	18.40	4.04	0.96	0.67
	2	20.55	16.27	3.36	0.37	0.55
	3	24.87	19.80	4.10	0.29	0.68
	4	18.23	14.53	2.94	0.28	0.48
	5	19.34	15.42	3.12	0.28	0.52
	6	22.30	17.29	3.67	0.74	0.60
	7	22.98	18.06	3.83	0.36	0.63
	mean	21.76	17.11	3.58	0.47	0.59
6	1	17.42	13.74	2.72	0.48	0.48
	2	23.32	18.20	3.75	0.71	0.66
	3	23.16	18.64	3.75	0.11	0.66
	4	23.43	17.96	4.57	0.09	0.81
	5	23.98	18.24	4.63	0.29	0.82
	6	17.53	14.04	2.79	0.21	0.49
	7	23.37	18.37	3.72	0.62	0.66
	mean	21.74	17.03	3.70	0.36	0.65
7	1	21.86	17.62	3.41	0.24	0.59
	2	17.24	13.69	2.66	0.44	0.45
	3	19.96	15.88	3.02	0.54	0.52
	4	26.20	21.20	4.14	0.14	0.72
	5	21.31	16.39	3.46	0.87	0.59
	6	20.86	17.01	3.18	0.12	0.55
	7	23.55	18.17	3.73	1.01	0.64
	mean	21.57	17.14	3.37	0.48	0.58
8	1	20.80	16.22	3.33	0.68	0.57
	2	18.91	15.67	2.71	0.07	0.46
	3	24.64	19.63	4.02	0.29	0.70
	4	24.86	19.09	4.06	1.01	0.60
	5	21.49	17.48	3.31	0.13	0.57
	6	25.65	20.57	4.16	0.20	0.72
	7	20.25	16.73	2.94	0.08	0.50
	mean	22.37	17.91	3.50	0.54	0.59
9	1	18.62	15.19	2.78	0.11	0.54
	2	17.77	14.02	2.68	0.55	0.52
	3	21.68	17.58	3.28	0.18	0.64
	4	19.76	16.34	2.78	0.10	0.54
	5	23.32	17.78	4.36	0.33	0.85
	6	22.22	18.21	3.22	0.17	0.62
	7	19.02	15.91	2.59	0.02	0.50
	mean	20.34	16.43	3.10	0.21	0.60

Length of Fast (weeks)	Fish Sample Number	Wet Weight (g)	Water Content (g)	Protein Content (g)	Lipid Content (g)	Residue* (g)
10	1	19.05	15.83	2.64	0.07	0.51
	2	19.43	16.15	2.69	0.07	0.52
	3	18.85	15.29	2.76	0.26	0.54
	4	21.63	18.10	2.88	0.09	0.56
	5	21.51	17.05	3.23	0.60	0.63
	6	20.31	17.00	2.69	0.10	0.52
	7	21.22	16.96	3.16	0.49	0.61
	mean	20.29	16.62	2.86	0.24	0.56

APPENDIX IV

RESULTS OF FITTING A THIRD DEGREE POLYNOMIAL EQUATION TO TEN WEEK FASTING SALMON DATA

Regression equation:

$$y = 17.23365 - 0.2103547x + 0.0439724x^2 - 0.004579616x^3$$

y = per cent protein of wet body weight
x = length of starvation in weeks + 1

Standard error of estimate: 0.806

Curvilinear correlation coefficient: 0.758

Length of Fast (weeks)	Wet Body Protein (%)	Change in Body Protein (%/week)	Loss of Nitrogen (mg/kg fish/day)	Protein Energy Release* (kcal/kg fish/day)	Ratio** (mg N/kcal total metabolism)
0	17.1				
1	17.0	0.1	23	0.66	1.9
2	16.9	0.1	23	0.66	2.1
3	16.8	0.1	23	0.66	2.3
4	16.7	0.1	23	0.66	2.5
5	16.6	0.1	23	0.66	2.8
6	16.3	0.3	69	2.0	8.0
7	16.0	0.3	69	2.0	9.0
8	15.6	0.4	91	2.7	12
9	14.9	0.7	160	4.7	19
10	14.1	0.8	180	5.4	22

*caloric equivalent of protein is taken to be 4.7 kcal/g

**total heat production is the sum of daily protein energy release and fat energy release (Table XXII)

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