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Dietary requirements of rainbow trout for tryptophan, lysine and arginine determined by growth and biochemical measurements

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Abstract

Three separate studies were performed to determine the dietary requirements of rainbow trout *Salmo gairdneri* for tryptophan (Trp), lysine (Lys) and arginine (Arg) from both growth and biochemical data. The growth studies were carried out over a 12 week period. From graphical plots of % mean weight gain against % amino acid in diet the following requirement values were obtained, Trp 0.25% diet (0.4% dietary crude protein); Lys 1.9% diet (4.3% dietary protein); and Arg 1.6–1.8% diet (3.6–4% dietary protein). Plasma and liver amino acid concentrations measured 20h after feeding did not prove useful for determination of requirement values. Hepatic activities of Trp pyrrolase (TP), Lys α ketoglutarate reductase (LKGR) and arginase were not significantly affected by varying levels of Trp, Lys and Arg respectively in the diet. TP has a cytosolic location and a Km of 0.2 mM for Trp; LKGR is mitochondrial and the Km for Lys is 7.3 mM; arginase is also mitochondrial and has a Km of 4.9 mM for arginine. Measurements of expired $^{14}CO_2$, after injection of a tracer dose of ^{14}C amino acid, did allow estimates of requirement levels to be made. The values obtained from the oxidation studies reinforced the values obtained from the growth data but were not precise enough to justify using this method on its own.

Introduction

In the formulation of diets for rainbow trout (*Salmo gairdneri*) the dietary requirement values for essential amino acids (EAA) are generally based upon the NRC (1981) values for chinook salmon (*Oncorhynchus tshawytscha*). These values were obtained from the growth data of fish fed graded levels of each amino acid in test diets containing an amino acid profile resembling hydrolysed whole egg protein except for the amino acid being tested. Similar growth studies have been performed using rainbow trout for some but not all EAA. From the evidence available so far, it appears that the requirements of trout and salmon are not necessarily the same. For example, Kaushik (1979) reported a lower requirement for arginine by trout than chinook salmon (1.4 compared to 2.4% diet). Ogino (1980) used a different technique to estimate requirement values for trout. He measured retention of EAA in the bodies of fish fed diets containing different proteins of high biological value. This method, however, did not take into account any requirements for body maintenance, and thus it was assumed that no dietary EAA were oxidized or transformed into other nitrogen compounds. It is,

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however, well known that only 40–50% of amino-N consumed is retained as amino-N in the carcass. The retention efficiency of EAA (compared to NEAA) is presently unknown but is likely to be 100%. The method of Ogino nevertheless gave comparable requirement values to those for chinook salmon for all EAA except arginine, methionine and phenylalanine.

The present work was carried out to determine the dietary requirements of rainbow trout for 3 EAA namely, tryptophan, lysine and arginine. In addition to the growth studies, attempts were made to determine requirement values by the measurement of some biochemical parameters (tissue and blood amino acid concentrations, oxidation of injected ^{14}C-amino acid and hepatic activities of enzymes which initiate amino acid catabolism). Such measurements have been used successfully in mammalian studies (e.g. Brooks *et al.* 1972; Lewis *et al.* 1977). These biochemical methods were used both to assess their suitability in fish studies and also to gain further evidence for the requirement values obtained from the growth data.

Materials and methods

Much of the data and experimental details relating to the studies on tryptophan and lysine requirements have been published previously (Walton *et al.* 1984a, b) and are not repeated here. The experimental details in this paper relate to the studies with arginine unless otherwise stated.

Fish and diets

Rainbow trout of approximately 7g mean weight were obtained from Selcoth fish farm, Moffat, Scotland. They were randomly distributed among 12 circular glass fibre tanks. Tank dimensions, water flow details etc. were as described previously (Walton *et al.* 1984b). Water temperature varied from 8–12°C during the course of the experiment.

The basic composition of the diets used in the 3 studies is shown in Table 1. The diets were prepared such that the final EAA compositions (apart from the test amino acid) simulated the amino acid composition of hydrolyzed cod muscle protein when present at a dietary level of 450 g protein/kg diet. The amino acid profiles of the diets were made up from a mixture of 2 "whole" proteins plus some crystalline amino acids. This procedure is shown in more detail in Tables 2 and 3 which give the composition of the arginine test diets. The dietary amino acid levels in the test diets for the 3 studies were thus, tryptophan 0.08, 0.13, 0.2, 0.4 and 0.6% diet; lysine 1, 1.2, 1.4, 1.7, 2, 2.3 and 2.6% diet; arginine 0.8, 1.3, 1.8, 2.3 and 2.8% diet.

Table 1. Basic composition of the diets (g/Kg diet) used in the dietary requirement studies

	Trp	Lys	Arg	Control
White fish meal[1]	120	120	120	530
Zein	–	–	300	–
Gluten	–	300	–	–
Gelatin	250	–	–	–
Starch[2]	160	200	190	200
Fish oil[3]	150	150	150	150
Vitamin mix[4]	29	29	29	29
Mineral mix[4]	40	40	40	40
EAA mix[5]	108	108	106	–
NEAA mix[5] + test AA	143	53	65	–
Cellulose	–	–	–	51

[1] 85% protein by Kjeldahl analysis; [2] Paselli WA4, Avebe, Veenram, Holland; [3] Fosol, Marfleet Refining Co., Hull, England; [4] Mineral and vitamin mixes as described by Cowey *et al.* (1981); [5] Essential amino acid mixes and non essential amino acid mixes (see Walton *et al.* (1984a, b) and Tables 2 and 3).

Each (arginine) diet was given 3 times daily to duplicate tanks of fish at a level of 5% body weight/day for 6 days each week. At this level of feeding the fish were effectively fed to satiation but there was some wastage of diet which is reflected in the lower than normal feed/gain ratios shown in Table 2. The diets were prepared as moist pellets which were then freeze dried and stored at −20°C until required. Fish were weighed individually every 4 weeks during the 12 week duration of the growth experiment. The fish were maintained on the same diets for a further 4 weeks during which the biochemical parameters were measured.

Table 2. Amino acid composition (g) of dietary basal mix (proteins + free amino acids)[1] contained in 1 kg complete diet for the arginine requirement studies

Amino acid	From 120 g white fish meal	+	From 300 g zein	=	Total from protein	Free amino acid added	Amount (g) in 450 g cod muscle protein
Alanine	5.4		25.1		30.5	1.9	32.4
Arginine	4.8		3.9		8.7	*[2]	30.3
Aspartic acid	7.5		14.5		22.0	*[2]	47.9
Cystine	0.6		1.1		1.7	4.7	6.4
Glutamic acid	11.1		6.2		17.3	*[2]	74.6
Glycine	6.6		3.0		9.6	13.3	22.9
Histidine	2.1		3.1		5.2	10.4	15.6
Isoleucine	3.5		11.6		15.1	6.8	21.9
Leucine	6.1		54.8		60.9	–	41.9
Lysine	6.2		0.4		6.6	39.8[3]	46.4
Methionine	2.6		4.8		7.4	1.9	9.3
Phenylalanine	3.3		19.9		23.2	–	21.0
Proline	3.8		26.6		30.4	–	18.9
Serine	4.0		14.7		18.7	*[2]	24.2
Threonine	3.7		7.5		11.2	12.5	23.7
Tryptophan	0.8		–		0.8	5.1	5.9
Tyrosine	2.8		14.3		17.1	1.1	18.2
Valine	3.8		9.1		12.9	13.2	26.1

[1] Total weight (in complete diet) of white fish meal + zein + free amino acids = 540.8 g; [2] Variable amounts of these amino acids were added to the basal mix to form the experimental diets (see Table 2); [3] Supplied as 49.9 g lysine monohydrochloride.

Table 3. Composition (g/kg dry diet) of the arginine experimental diets

Component	Diet				
	1	2	3	4	5
Basal mix[1]	540.8	540.8	540.8	540.8	540.8
Amino acid mix[2]	50.3	46	41	36	31
Arginine	0	4.3	9.3	14.3	19.3
Starch	190	190	190	190	190
Cod liver oil	150	150	150	150	150
Vitamin mix[3]	28.9	28.9	28.9	28.9	28.9
Mineral mix[3]	40	40	40	40	40
Total arginine	8.7	13	18	23	28

[1] Basal mix (see Table 2); [2] The amino acid mix was prepared by mixing together (g): 67.6 aspartic acid, 14.3 serine, 148.2 glutamic acid; [3] Composition of the mineral and vitamin mixes were as described previously (Cowey *et al.* 1981). 0.4 g ascorbyl palmitate and 0.5 g butylated hydroxyanisole were added to the vitamin mix.

Amino acid analyses

Blood and liver samples were collected 18h after feeding since this was found to be the optimal sampling time by Kaushik (1979). Fish were anaesthetised with m-amino benzoic acid methyl sulphonate (1 g/10 l water) and blood collected from the caudal vein using heparinised syringes. Red blood cells were removed by centrifugation (10,000 g for 5 min) and the plasma deproteinised by the addition of 3 vol. 0.2 M 4-sulphosalicyclic acid. Livers were deproteinised by homogenising in 4 vol. of the same solution. Protein precipitates were removed by centrifugation and the resultant supernatants stored at −20°C. Amino acids in the samples were resolved on a Jeol amino acid analyser (model JLG 6AH) and peak areas were automatically calculated by a Shimadzu CR1-A peak height integrator.

Samples of diets and dietary components were hydrolysed in 5.7 M HCl by the procedure of Roach *et al.* (1967) before amino acid analysis. Tryptophan in dietary components was analysed after alkaline hydrolysis in 5 M NaOH by the procedure of Basha and Roberts (1977).

Oxidation of L (U-^{14}C) arginine

L (U-^{14}C) arginine (336 μCi/μmole) was obtained from Amersham International, U.K. A solution of this isotope was prepared in 0.15 M NaCl such that 50 μl contained 1 μCi. One μCi was injected intraperitoneally at 1300 h and the CO_2 expired between then and 0900 h the following day collected by the method described previously (Walton *et al.* 1984b).

Arginase assay

Arginase was determined at 15°C by the method of Portugal and Aksnes (1983). Assays were carried out on suitably diluted preparations for 10 min following disruption of the mitochondrial membranes by 5 × 20 sec bursts of sonication. For the intracellular localization studies liver and kidney tissues were fractionated as described previously (Walton and Cowey 1979) except that 0.25 M sucrose was replaced by 0.25 M mannitol since sucrose interferes with the enzyme assay (Mora *et al.* 1965). Glutamate dehydrogenase (Schmidt 1974) and lactate dehydrogenase (Bergmeyer *et al.* 1963) were assayed as marker enzymes for the mitochondrial and cytosolic fractions respectively. For the Km determinations the enzyme assay was modified slightly by the addition of 0.5 ml 1 M sodium glycinate buffer pH 9.5 to all incubations.

Results

Mean weight gains, feed conversion ratios, specific growth rates and mortalities for fish fed the arginine test diets are shown in Table 4. No gross pathology or deaths due to the diets were observed. In contrast tryptophan deficiency caused extensive cataract formation, spinal deformations (scoliosis) and increased levels of Ca, K and Na in the kidney; lysine deficiency caused some fin erosion. All fish given diets containing amino acid / protein mixtures had inferior weight gains and feed conversion ratios to those fish given the control diet which contained only "whole" protein as its source of amino acids.

When a conventional Almquist (1972) type plot of mean weight gain against dietary arginine level was drawn, by eye, (Fig. 1) the "breakpoint", which indicates the dietary requirement, was in the range 1.6–1.8% diet (equivalent to 3.6–4% dietary crude protein). From similar type plots (Fig. 1) the dietary requirements for tryptophan and lysine were found to be 0.5% diet (0.44% dietary crude protein) and 1.9% diet (4.3% dietary crude protein) respectively.

It did not prove possible to determine requirement values from the plots of either blood/plasma or liver amino acid concentrations against dietary amino acid level (Fig. 2). When this method was used to determine the requirement of rats for tryptophan and lysine (Lewis *et al.* 1977) the plasma amino acid concentration remained low in rats fed sub-requirement levels of the amino acid. But when adequate levels of the amino acid were fed the

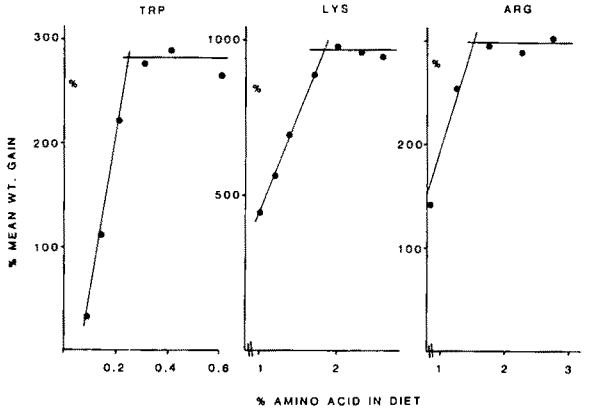

Fig. 1. Graphical plots of % mean weight increase against amino acid concentration (% by weight) in the diet for the 3 experimental studies.

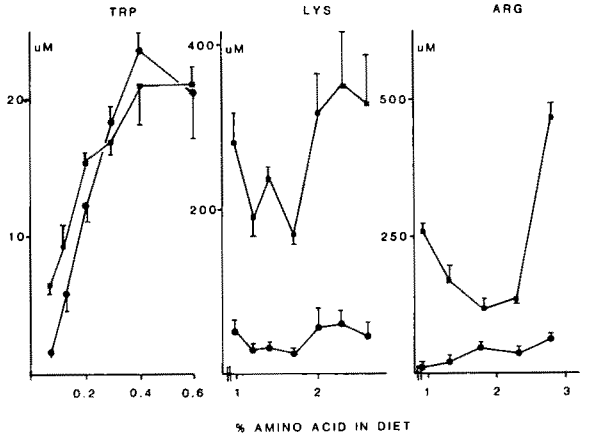

Fig. 2. Graphical plots of plasma (●) and liver (■) amino acid concentrations (μM) against amino acid concentration in the diet for the 3 experimental studies.

Note: Values shown are means ± SE from 5 determinations for Trp, 6 for Lys and 3 for Arg.

plasma concentrations tended to increase linearly as the dietary concentration rose. However in no case was such a pattern clearly seen in these studies with rainbow trout. It may be that sampling time is critical and the use of a different time could have produced a more useful result.

Plots of % dose injected expired as CO_2 against dietary amino acid level were more useful in determining requirement values (Fig. 3). As with plasma amino acid concentrations one might expect oxidation of the amino acid to be low in fish fed sub-requirement levels and to increase once the require-

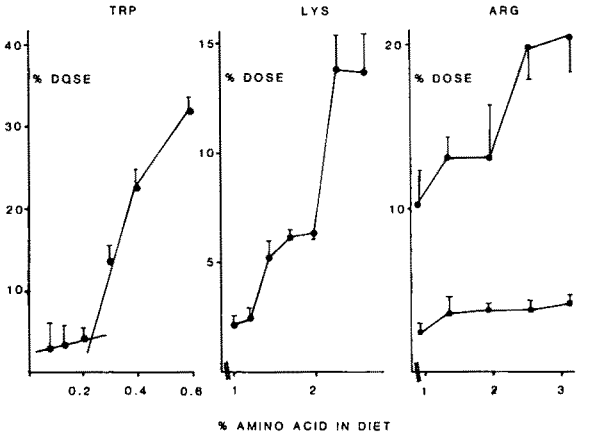

Fig. 3. Graphical plots of % dose of injected ^{14}C amino acid expired as CO_2 over a 20h period against dietary amino acid concentration for the 3 experimental studies.

Note: Values shown are means ± SE from 5 determinations for Trp and Lys and 5 for Arg. Also shown is the residual radioactivity in the water (■) for the arginine study.

ment level had been met. This pattern was most clearly seen in the results from the use of (1 – ^{14}C) tryptophan and a requirement level of 0.21% diet was indicated (compared to 0.25% from the growth data). The results using (U-^{14}C) lysine and (U-^{14}C) arginine were not so clearly defined. Nevertheless regions where large increases in oxidation occurred can be seen in the graphical plots. These indicated requirement values of between 2–2.4% diet (compared to 1.9% from the growth data) for lysine and between 1.8–2.3% diet (compared to 1.6–1.8% from the growth data) for arginine. Residual radioactivity (after removal of expired CO_2 by acidification) in the water the test fish were kept in was low (0.5–1% dose) in the studies with tryptophan and lysine. Higher levels (2.5–4.3% dose) were found in the arginine study. No attempts were made to identify the radioactive substances in the water; they were presumed to be mainly excretory products including urea.

Before measuring the effects of diet on the hepatic activities of amino acid catabolising enzymes some preliminary experiments were conducted. Tryptophan pyrrolase EC 1.13.11.11 was only detected in liver where it had a cytosolic location and a Km of 0.2 mM for tryptophan. Lysine-α ketoglutarate reductase EC 1.5.1.8 had a

Table 4. Growth data of rainbow trout (*Salmo gairdneri*) given diets[1] containing different levels of arginine for 12 weeks

Diet no.	1	2	3	4	5	Control
Mean initial wt (g)[2]	7.31	7.23	7.41	7.30	7.24	6.74
Mean final wt (g)[2]	17.73	25.82	29.26	28.45	29.53	42.45
Mean wt gain (%)	142.5	257.3	294.7	290.2	306.3	529.7
Specific growth rates[3]	1.06	1.52	1.64	1.67	1.67	2.19
Feed conversion ratio[4]	3.33	2.22	2.01	2.00	1.96	1.36
Mortalities	0	0	0	1	0	0

[1] For details of diets, see Table 3; [2] Values are the average of the mean values obtained from each of the two tanks of fish per treatment (30 fish per tank); [3] 100 (ln (final weight) − ln (initial weight))/84 d; [4] dry weight feed/wet weight gain (mean values for each pair of tanks per diet treatment).

Table 5. Intracellular distribution of arginase (EC 3.5.3.1) in rainbow trout (*Salmo gairdneri*) liver and kidney

Enzyme	Tissue	Fraction				
		Homogenate	Nuclear	Mitochondrial	Cytosolic	Microsomal
Arginase	Liver	100	2.4 ± 0.7	97.1 ± 2.7	3.5 ± 1.1	1.0 ± 0.5
	Kidney	100	24.1 ± 7.3	75.4 ± 4.6	14.3 ± 0.7	1.7 ± 0.4
Glutamate	Liver	100	2.1 ± 0.4	94.1 ± 3.0	1.9 ± 0.3	1.5 ± 0.1
Dehydrogenase	Kidney	100	13.0 ± 3.2	71.1 ± 5.1	10.0 ± 2.3	5.0 ± 0.5
Lactate	Liver	100	0.6 ± 0.1	1.8 ± 0.5	102.0 ± 6.7	0.9 ± 0.2
Dehydrogenase	Kidney	100	5.6 ± 2.0	1.1 ± 0.6	97.1 ± 2.0	1.0 ± 0.3

Values are means ± SE (for 3 fish given a commercial diet) and are expressed as % activity found in whole homogenate.

mitochondrial location in the liver and a Km of 7.3 mM for lysine and a Km of 0.5 mM for α ketoglutarate. Arginase EC 3.5.3.1 is present in relatively high levels in both liver and kidney of fish (Cvancara 1969). In both these tissues arginase has a predominantly mitochondrial location (Table 5). Teleost kidney consists of an anterior (head) region, which is composed principally of haemopoietic tissue, and a posterior region, which is mainly concerned with excretion. In some species these two regions are separate but in trout they are fused so it is difficult to obtain pure preparations of them. However when trout kidneys were divided into 3 equal portions (by length) the relative activities of arginase (measured in μmol/min/g wet weight) were 1:7:10 for the head:middle:tail regions. Thus in kidney arginase is chiefly associated with the excretory regions. Km values for arginine were similar for both liver (4.9 mM) and kidney (4.6 mM) enzymes. In fish fed the various test diets no significant effects due to dietary amino acid concentration were seen on the activities in liver of any of the 3 enzymes measured (Table 6). Hence it was not possible to determine requirement values from these results.

Discussion

The dietary requirement values (obtained from the growth data) of rainbow trout for the 3 amino acids tested in these studies are similar to a number of other reported values for salmonids. Thus the value of 0.25% diet for tryptophan compares with the 0.2% diet found for chinook salmon (NRC 1981) and trout (Ogino 1980). The value of 1.9% diet for lysine compares to 2.1% for trout (Ogino 1980), 2.0% for chinook salmon (NRC 1981) and 1.9% for chum salmon (Akiyama *et al.* 1985) but disagrees with the values of 2.9% and 1.3% found

by Ketola (1983) and Kim and Kayes (1983) respectively for trout. The value of 1.6–1.8% diet for arginine is lower than the 2.4% for chinook salmon (NRC 1981) and 2.5–2.8% for trout (Ketola 1983) but is of a similar order to other values in (sometimes preliminary) reports with trout of 1.4–1.6% (Ogino 1980; Kim *et al.* 1983; Cho and Woodward 1985; Kaushik and Fauconneau 1985). While variations among different laboratories are to be expected some of the differences encountered are considerable. It is held that there is a constant relationship between amino acid requirement and protein intake up to the protein level required for maximum growth (Almquist 1972). However some of the reported differences in amino acid requirements are still apparent if the values are expressed as % dietary crude protein rather than % diet.

Trout given diets containing amino acid/protein mixtures grow less well than when complete proteins are fed. This phenomenon has also been observed in other species (see Cowey and Luquet 1983) but the reasons for the growth differences have not been fully explained. One possible reason could be the different rates of uptake from the gut of amino acids fed in a free form compared to those fed as components of protein. This could affect the concentrations of the amino acids at the sites of protein synthesis and sites of degradation. Alternatively, Murai *et al.* (1981) have suggested that absorption by mucosal cells of small peptides, resulting from protein hydrolysis, is very efficient and may lead to better utilisation of amino acids than when they are present in the free form. Hence, some reservations over values obtained from the use of diets containing considerable amounts of free amino acids must remain until it is possible to achieve growth rates more similar to those obtained with "whole" proteins. However, there is no evidence to show that such values are inherently inaccurate and those obtained from the current studies are similar to those obtained by Ogino (1980) using a different technique.

Of the biochemical parameters measured in order to reinforce the values obtained from the growth data only the oxidation data proved to be of use. The rationale behind these techniques has been described previously (Brookes *et al.* 1972; Lewis *et al.* 1977). The measurement of plasma and tissue amino acid concentrations has been used in other fish studies in attempts to determine dietary requirements. Sometimes this technique has met with success (Harding *et al.* 1977; Kaushik 1979; Wilson *et al.* 1977, 1978) and other times with failure (Kaushik and Fauconneau 1985; Robinson *et al.* 1980, 1981; Wilson *et al.* 1980). Some of these differences are no doubt due to the amino acid being assayed. However, the same group has had both success and failure with this technique in separate studies with lysine (Wilson *et al.* 1977; Robinson *et al.* 1980). There is no ready explanation for these findings.

Measurements of $^{14}CO_2$ production from an injected dose of the relevant ^{14}C amino acid allowed estimates of requirements to be made. The clearest results were obtained in the tryptophan studies. This may be because it was possible to purchase commercially 1-^{14}C labelled amino acid whereas U-^{14}C labelled lysine and arginine had to be used. It is preferable to use a 1-^{14}C rather than U-^{14}C labelled amino acid because there are less involvements in side reactions and reuse of label etc. Despite this the results obtained were of a similar order to the values obtained from the growth data. However, this technique would not appear to be suitable for use in the absence of growth data because of the lack of precision in determining requirement values from the graphical plots. It is, on the other hand, useful to reinforce the values from the growth data.

The activities of the enzymes which initiate the catabolism in liver of tryptophan, lysine and arginine were little affected by variations in dietary content of the corresponding amino acid. Thus there appears to be no evidence for any adaptative response which would reduce catabolism of the amino acid when the trout were fed deficient diets. This situation can be compared to the similar lack of adaptative response of amino acid catabolising enzymes in the livers of fish fed diets containing either high or low levels of protein (Rumsey 1981). The catabolism of amino acids in carnivores such as trout appears to be permanently set to deal with its natural diet (high protein/low carbohydrate).

The intracellular distributions of tryptophan

Table 6. Effect of dietary amino acid level on the activities of amino acid catabolising enzymes in liver

1) *Tryptophan pyrrolase (5)*[1]							
% Trp in diet	0.08	0.13	0.2	0.3	0.4	0.6	
Activity[2]	0.009	0.022	0.015	0.026	0.024	0.021	
2) *Lysine ketoglutarate reductase (4)*							
% Lys in diet	1.0	1.2	1.4	1.6	2.0	2.3	2.6
Activity	0.16	0.16	0.16	0.17	0.18	0.17	0.18
3) *Arginase (6)*							
% Arg in diet	0.8	1.3	1.8	2.3	2.8		
Activity	69	61	67	55	72		

[1] Figures in parentheses are the number of separate determinations; [2] Enzyme activity is expressed as μmol/min/g wet weight liver at 15°C. Mean values are given in the table. Pooled SE for the activities were tryptophan pyrrolase 0.004; lysine ketoglutarate reductase 0.03; arginase 7.

pyrrolase and of lysine α ketoglutarate reductase are similar in both trout and mammals. In ureotelic mammals arginase is cytosolic and is mainly found in liver; in uricotelic birds arginase is mitochondrial and mainly found in the kidney. In ammoniotelic trout arginase has a mitochondrial location (Table 5) and is present in both liver and kidney (Cvancara 1969). The significance of these inter species differences is probably related to the presence or absence of a urea cycle. All the enzymes of the urea cycle have been detected in salmonid livers (Huggins *et al.* 1979) but some of them are present at such low levels that it is considered unlikely that a functional urea cycle exists in trout. Urea may still be produced in trout through the action of arginase on arginine or from purine degradation.

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