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Post Prandial Plasma Free Arginine Concentrations Increase in Rainbow Trout Fed Arginine-deficient Diets

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ABSTRACT : Three experiments were conducted to determine the effects of dietary arginine concentrations on plasma free amino acid (PAA) concentrations in rainbow trout, *Oncorhynchus mykiss* (Walbaum). The first experiment was conducted to determine appropriate post-prandial and food deprivation sampling times in dorsal aorta cannulated rainbow trout averaging 519±9.5 g (mean±SD) at 16°C. Blood samples were taken at 0, 2, 3, 4, 5, 6 and 24 h after feeding (0 and 24 h blood samples were taken from the same group of fish). PAA concentrations increased by 2 h post-feeding and the concentration of all essential amino acids except histidine peaked at 5 h and returned to 0 time values by 24 h. In the second experiment dorsal aorta cannulated rainbow trout averaging 528±11.3 g (mean±SD) were divided into 6 groups of 4 fish to study the effect of dietary arginine levels on PAA. After 24 h food deprivation, each group of fish was fed one of six L-amino acid diets containing graded levels of arginine (0.48, 1.08, 1.38, 1.68, 1.98 or 2.58%) by intubation. Blood samples were taken at 0, 5 and 24 h after feeding. Post-prandial (5 h after feeding) plasma-free arginine concentrations (PParg) showed a breakpoint at 1.03% arginine in the diet and post-absorptive (24 h after feeding) plasma free-arginine concentrations (PAarg) showed a breakpoint at 1.38% arginine. PAarg increased linearly from fish fed diets containing arginine between 0.48% and 1.38%, and the concentrations remained constant from fish fed diets containing arginine at or above 1.38%, but were all below PParg at all time points. Results of the third experiment confirm the results that PParg concentrations from fish fed arginine deficient diets were higher than PAarg (0 or 24 h values). Thus, in contrast to mammals and birds, the PParg when arginine is present in the diet as the most limiting amino acid such that it severely limits growth, increases in plasma rather than decreases. *(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 3 : 396-402)*

Key Words : Arginine, Rainbow Trout, Dorsal Aorta Cannulation, Plasma Free Amino Acids

INTRODUCTION

Evaluation of plasma free amino acids concentrations in mammals and birds has led to discoveries involving the genetic defects of amino acid metabolism, the secondary perturbations of amino acid metabolism as a result of primary renal or liver disease and the effects of amino acid deficiencies, imbalances and toxicities on amino acid metabolism (Zicker and Rogers, 1990). Factors influencing plasma free amino acids (PAA) concentrations in growing animals have been studied extensively. Although assay procedures used in PAA studies have varied considerably, the results obtained have been quite consistent in demonstrating that dietary amino acid deficiencies result in reduced plasma concentration of that amino acid, post-prandially (Hill and Olsen, 1963), whereas dietary amino acid excesses have resulted in increase of that amino acid in the plasma (Richardson et al., 1953).

Studies in the chick (Richardson et al., 1953; Hill and Olsen, 1963), rat (Swendseid et al., 1963; Young and Zamora, 1968), pig (Puchal et al., 1962), human (Longenecker and Hause, 1961; Snyderman et al., 1964) and fish (Thebault, 1985) have clearly established that a reduced concentration of an essential amino acid (EAA) in plasma reflects a deficient level of that amino acid in the diet. Others (Munro, 1970; Young and Scrimshaw, 1970; Young et al., 1971) showed that the pattern of amino acids and the level of a specific EAA in plasma correlate with the ability of the dietary protein to support growth. The relationships between the concentration of PAA and dietary amino acid intake have been the subject of reviews (Leathem, 1968; McLaughlan and Morrison, 1968; Munro, 1970; Young and Scrimshaw, 1970; Zicker and Rogers, 1990).

Although the effects of dietary protein sources and amino acid mixtures on plasma free essential amino acid concentrations in sea bass (Thebault, 1985) and in rainbow trout (Schuhmacher et al., 1997; Vermeirssen et al., 1997) have been reported, the complete dose-response relationships for arginine have not been investigated Therefore, the objectives of the present study were to

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Table 1. Composition of the basal diet (% of dry matter)[1]

Ingredients	%
EAA[2]	17.27
NEAA[3]	12.36
Casein[4]	5.00
Gelatin[4]	2.00
Dextrin[4]	27.97
Dextrose[4]	5.00
α-cellulose[4]	8.20
Fish oil[5]	10.00
Carboxymethyl cellulose[4]	1.00
$Ca(H_2PO_4)_2H_2O$	3.00
Choline bitartrate[4]	1.20
Vitamin mixture[6]	3.00
Mineral mixture[7]	4.00

[1] Diets were neutralized with NaOH to give a final pH 6.6.
[2] EAA: Essential amino acids, Ajinomoto, Tokyo, Japan.
[3] NEAA: Non-Essential amino acids, Ajinomoto, Tokyo, Japan.
[4] United States Biochemical (USB), Cleveland, Ohio.
[5] Ewha Oil Company, Pusan, Korea.
[6] Vitamin mixture (mg/kg feed unless indicated otherwise): vit. A, 3,000 IU; vit. D_3, 2,400 IU; vit. E, 120 IU; menadione sodium bisulfate, 6; vit. B_1-HCl, 15; vit. B_2, 30; vit. B_6-HCl, 15; vit. B_{12}, 0.06; vit. C, 300; calcium pantothenate, 150; nicotinamide, 150; inositol, 150; d-biotin, 1.5; choline chloride, 3,000; pancreatin, 12.5. Vitamin mixture prepare by our laboratory and the individual vitamins purchased from USB, Cleveland, Ohio, USA.
[7] Mineral mixture (mg/kg feed): $MnSO_4$, 320; $ZnSO_4$, 270; $FeSO_4$, 750; $CuSO_4$, 60; $CoSO_4$, 7; $MgSO_4$, 17.3; K_2SO_4, 212; NaCl, 519; K_2HPO_4, 136; $NaSeO_3$, 0.01; KI, 0.15. Mineral mixture was prepared by our laboratory and the individual minerals purchased from Junsei Chemical, Tokyo, Japan.

Table 2. Amino acid composition of the basal diet (%of dry matter)

Amino acids	From casein +gelatin	From crystalline amino acids	Total[1]
EAA			
Arginine	0.353	1.924[2]	2.277
Histidine	0.194	0.725	0.919
Isoleucine	0.252	1.674	1.926
Leucine	0.493	2.702	3.195
Lysine	0.502	1.904	2.406
Methionine	0.152	1.030	1.182
Cystine	0.019	0.172	0.191
Phenylalanine	0.271	1.742	2.013
Tyrosine	0.270	1.335	1.605
Threonine	0.221	1.601	1.822
Tryptophan	0.065	0.462	0.527
Valine	0.350	1.999	2.349
NEAA			
Alanine	0.345	1.741	2.086
Aspartic acid	0.483	3.280	3.763
Glycine	0.538	0.758	1.296
Glutamic acid	1.298	3.616	4.914
Proline	0.790	0.568	1.358
Serine	0.374	2.398	2.772

[1] The amino acid profile simulated that of 35% whole chicken egg protein (Robinson et al., 1981).
[2] Six experimental diets were formulated to have graded levels of arginine (0.48, 1.08, 1.38, 1.68, 1.98 or 2.58%); equal amounts of aspartic acid and glutamic acid by weight were substituted by arginine in the basal diet.

determine the effects of the different dietary arginine levels on PAA concentrations and to estimate the dietary arginine requirement by using surgically modified young growing rainbow trout (*Oncorhynchus mykiss*).

MATERIALS AND METHODS

Animals and husbandry

Rainbow trout averaging 519±9.5 g (Experiment I), 528±11.3 g (Experiment II) and 521±13.1 g (Experiment III) were obtained from Ewhajung Trout Farm in Sang Joo, Korea. For all experiments, net cages (1.3 m×1.3 m×1.3 m) were placed in a flow-through raceway with a water flow of 60 L/min. Supplemental aeration was also provided to maintain the dissolved oxygen near 7.2±0.4 mg/L. Water temperature was maintained at 16±0.2°C.

Dorsal aorta cannulation and intubation

The trout were anesthetized with 200 mg/L 3-aminobenzoic acid ethyl ester methansulfonate (MS 222, Sigma Chemical Company, St. Louis, MO) for 3 to 5 minutes, placed on a V-shape table and gills were continuously irrigated with 16°C water containing 100 mg/L of MS 222 during the operation. A 50 cm-long cannula (Clay Adams PE 50 tubing, Parsippany, NJ) with a bubble about 5-6 cm from one end was washed with heparinized Cortland saline solution (Houston, 1990) and a 13-gauge needle was used to pierce a hole on the right nostrum (ventral side up) for the cannula to exit. A 19-gauge needle was used to bore a small hole in the roof of the mouth at the mid-line behind the third gill arc at a 30° angle and a piano wire was inserted into the PE 50 tubing as a guide. The proper insertion was verified by the observation of a slow blood flow after the wire was withdrawn from the cannula. A 3 ml syringe with a 23-gauge needle was used to remove air and blood clot and the cannula was flushed with the heparin solution. The cannula was sutured behind the bubble on the roof of the mouth, led out from the right nostrum, plugged with a color head pin, and sutured at the dorsal fin (I-H Ok et al., 2001; Bai et al., 2003).

Experimental design and diets

Experiment I was conducted to determine the appropriate post-prandial and post-absorptive time for blood sampling in dorsal aorta cannulated rainbow trout (I-H Ok et al., 2001; Bai et al., 2003). After dorsal aorta cannulation, the trout were divided into 6 groups (4 fish per group) in each net cage and were fed a commercial rainbow

trout diet (Woosung Feed Co. Ltd., Taejon-Si, Korea) for 3 days until the fish recovered from the operation of dorsal aorta cannulation. After 24 h food deprivation, these fish were intubated with the L-amino acid based diet at 1% body weight (dry-matter), anesthetized with MS222 and blood was sampled at 0, 2, 3, 4, 5, 6 and 24 h thereafter (0 and 24 h blood samples were taken from the same group of fish). The basal diet was formulated by the modification of Kim (1997) and contained a 29.6% crystalline amino acid mixture, 5% casein and 2% gelatin. Ingredients and amino acid composition of the basal diet are shown in Table 1 and 2, respectively. The ingredient mixtures without oil were stored at -80°C until used and basal diet was prepared by adding fish oil (10% of diet) and water (0.4 part of distilled water:diet, w/w) before intubation.

Experiment II was conducted to determine the effects of different dietary arginine levels on post-prandial (5 h after feeding, PParg) and post absorptive (24 h after feeding, PAarg) plasma free arginine concentrations in rainbow trout. Rainbow trout were divided into 6 groups of 4 fish each in net cage and fed a commercial diet (Woosung Feed Co. Ltd., Taejon-Si, Korea) for 3 days until the fish recovered from the operation of dorsal aorta cannulation. After 24 h food deprivation, these fish were intubated with 1% body weight (dry-matter) of the experimental diets. Six diets were formulated to contain 0.48, 1.08, 1.38, 1.68, 1.98 or 2.58% of arginine. Equal amounts of aspartic acid and glutamic acid by weight were substituted for the proper amounts of arginine in the diets. Each group of fish was anesthetized and blood was sampled from each fish within a group at 0, 5 and 24 h after intubating the experimental diets (0.4 parts of distilled water:diet, w/w) by using a 3 ml syringe.

Experiment III was conducted to confirm the results from Experiment II in which PParg were higher than PAarg from fish fed the arginine deficient diet (0.48%). Four rainbow trout were fed a basal diet for 3 days until the fish recovered from the operation of dorsal aorta cannulation. After 24 h food deprivation, these fish were intubated at 1% body weight (dry-matter) of the 0.48 or 2.58% arginine diet. Two consecutive dietary periods were used: the first period, 3 days of the 2.58% arginine diet; the second period, 3 days of the 0.48% arginine diet. In the first period, 0 h post feeding blood samples were taken at the beginning of day 1 and 24 h later (24 h after feeding). During the second period, 0 h and postprandial (5 h after feeding) blood samples were taken for 3 days (day 1, 2 and 3). The post-absorptive (0 h) blood samples were taken on day 4 (24 h after feeding fish on day 3).

Sample collection and analysis

Fish were anesthetized with 200 mg/l MS222 and 300 µl blood were sampled from each fish. Plasma samples were prepared by centrifugation at 3,000×g for 10 min. For deproteinization, the plasma samples were mixed with 10%

Table 3. Plasma free amino acid concentrations (nmol/ml) after feeding the basal diet (Experiment I)[1]

Amino acids	Time (h) after feeding							Pooled
	0	2	3	4	5	6	24	SEM
EAA								
Arginine	79^{c}	131^{b}	119^{b}	128^{b}	228^{a}	78^{c}	131^{b}	9
Histidine	114^{d}	244^{a}	186^{b}	143^{c}	185^{b}	96^{d}	113^{d}	10
Isoleucine	84^{e}	200^{d}	260^{c}	346^{b}	488^{a}	367^{b}	110^{e}	26
Leucine	146^{f}	307^{e}	392^{d}	516^{c}	784^{a}	581^{b}	166^{f}	41
Lysine	108^{e}	270^{d}	354^{c}	422^{b}	515^{a}	237^{d}	248^{d}	24
Methionine	49^{d}	149^{c}	155^{c}	222^{b}	321^{a}	235^{b}	62^{d}	17
Phenylalanine	93^{e}	178^{d}	225^{c}	426^{b}	714^{a}	239^{c}	98^{d}	40
Threonine	123^{e}	432^{c}	532^{b}	450^{c}	793^{a}	518^{b}	165^{d}	41
Tryptophan	10^{d}	23^{ab}	26^{a}	25^{a}	20^{bc}	18^{c}	10^{d}	1
Valine	222^{c}	530^{b}	594^{b}	560^{b}	783^{a}	579^{b}	238^{c}	37
Total	1,028^{e}	2,464^{d}	2,843^{c}	3,238^{b}	4,831^{a}	2,948^{c}	1,341^{e}	181
NEAA								
Alanine	603de	1086^{b}	1248^{a}	715^{cd}	845^{c}	671^{d}	465^{e}	52
Aspartic acid	474^{e}	627^{d}	694^{c}	795^{b}	976^{a}	892^{ab}	512^{e}	36
Asparagine	103^{c}	165^{b}	185^{a}	153^{b}	156^{b}	98^{c}	115^{c}	8
Citrulline	38^{bc}	45^{b}	40^{b}	49^{a}	37^{c}	31^{d}	18^{e}	2
Glycine	329^{b}	446^{a}	241^{c}	143^{d}	140^{d}	242^{c}	396^{ab}	23
Glutamic acid	272^{d}	463^{c}	614^{b}	454^{c}	1,092^{a}	614^{b}	261^{d}	52
Ornithine	111^{b}	105^{b}	126^{a}	131^{a}	118^{ab}	70^{c}	48^{d}	8
Serine	112^{c}	253^{b}	255^{b}	282^{b}	377^{a}	415^{a}	134^{c}	21
Tyrosine	39^{e}	88^{d}	136^{c}	195^{b}	300^{a}	175^{b}	57^{e}	16
1-Methylhistidine	33^{e}	76^{c}	88^{c}	111^{b}	127^{a}	87^{c}	53^{d}	6
3-Methylhistidine	31^{d}	54^{c}	72^{c}	119^{a}	115^{a}	101^{b}	39^{d}	5

[1] Values are means of four fish where the means in each row with different superscripts are significantly different ($p<0.05$).

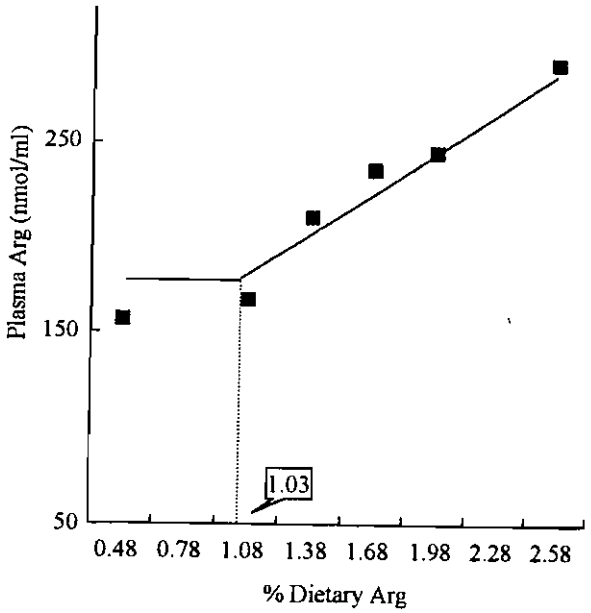

Figure 1. Plasma free arginine concentrations (nmol/ml) at 5 h (post-prandial arginine) after feeding in fish fed graded levels of dietary arginine (Experiment II). Y=175.1-33.2 (R-X), R=1.03± 0.299 (SE).

Table 4. Post-prandial and post-absorptive plasma free arginine concentrations (nmol/ml) of rainbow trout fed graded levels of dietary arginine (Experiment II) [1]

Level of arginine (%)						Pooled
0.48	1.08	1.38	1.68	1.98	2.58	SEM[2]
Post-prandial values						
157[c]	168[c]	211[b]	236[b]	245[b]	292[a]	1.98
Post-absorptive values						
66[d]	93[c]	117[ab]	113[b]	121[ab]	132[a]	0.82

[1] Values are means (n=5) and means with different superscripts are significantly different ($p<0.05$).
[2] Pooled standard error of mean: $SD/\sqrt{n}$.

5-sulfosalicylic acid in the ratio of four to one, cooled on ice for 30 min and centrifuged. The protein-free supernatant was diluted in pH 2.2 lithium citrate sample dilution buffer in the ratio of one to one and the samples were stored at -80°C until analysis. The plasma free amino acids were separated and quantified using a S433 amino acid analyzer (Sykam, Germany) using the ninhydrin method.

Statistical analysis

Data were subjected to analysis of variance test by using Statistix 3.1 (Analytical Software, St. Paul, MN, USA). When a significant treatment effect was observed, a Least Significant Difference test was used to compare means. Treatment effects were considered significant at $p<0.05$. The breakpoints for both PParg and PAarg were estimated by using the broken line model of Robbins et al. (1979).

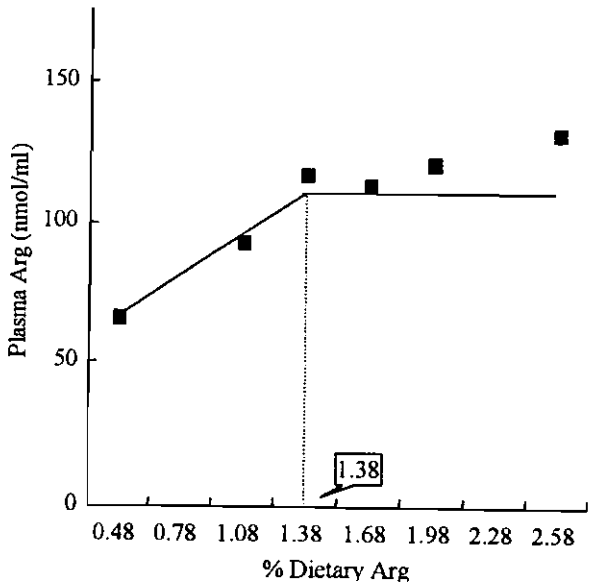

Figure 2. Plasma free arginine concentrations (nmol/ml) at 24 h (post-absorptive arginine) after feeding in fish fed graded levels of dietary arginine (Experiment II). Y=175.1-33.2 (R-X) R=1.03± 0.299 (SE).

RESULTS

Experiment I

Plasma free amino acid concentrations from fish force-fed the basal diet are shown in Table 3. Plasma free essential amino acid concentrations, with exception of histidine, lysine and tryptophan, began to increase at 2 h, peaked at 5 h and returned to near basal level at 24 h. Plasma free histidine concentration peaked at 2 h and returned to the basal level at 6 h. Plasma free lysine concentration peaked at 5 h and did not return to the basal level by 24 h. Plasma free tryptophan concentrations peaked at 2 h, remained constant between 2 h and 4 h and returned to the basal level at 24 h. Total plasma free essential amino acid concentrations peaked at 5 h and returned to near basal level at 24 h. Plasma dispensable amino acid concentrations peaked between 2 h and 6 h and returned to near basal levels at 24 h.

Experiment II

Post-prandial plasma free arginine concentrations (PParg) and post-absorptive plasma free arginine concentrations (PAarg) of fish fed graded levels of arginine are shown in Figure 1 and 2. PParg concentrations declined with decreasing dietary arginine. PParg from fish fed 0.48 and 1.08% arginine diets were not significantly different ($p>0.05$); however, PParg increased with dietary arginine levels among fish fed diets containing from 1.08 to 2.58%. PAarg significantly increased with dietary arginine levels

Table 5. Plasma free arginine concentrations (nmol/ml) from fish fed diets containing 0.48 and 2.58% dietary arginine (Experiment III)[1]

Dietary arginine (%)	Time (h) after feeding				
	0 h[2]	5 h (d1)	5 h (d 2)	5 h (d 3)	24 h (d 4)
0.48		153±25[a]	158±21[a]	168±34[a]	87±18[b]
2.58	143±27[a]				135±33[a]

[1] Values are means±SD from four fish and means with different superscripts are significantly different ($p<0.05$).

[2] 5 h postprandial blood samples were taken daily for 3 days (d1, 2 and 3) and 24 h post-absorptive blood samples were taken on day 4 (at 24 h after feeding fish on d 3). 0 h post-prandial blood samples were taken at the beginning of day 1 and feeding fish once a day, 24 h post-absorptive feeding blood samples were taken on day 4 (at 24 h after feeding fish on day 3).

from 0.48% to 1.38%, while it increased at a lower rate beyond 1.38%. The breakpoints, using the broken-line model, were 1.03 and 1.38% for PParg and PAarg, respectively.

Experiment III

There were no significant differences between PParg from fish fed 0.48% arginine diet and PAarg from fish fed 2.58% arginine diet. However, the fourth day PAarg from fish fed 0.48% arginine diet was significantly lower than PParg from fish fed the same diet and the PAarg from fish fed 2.58% arginine diet ($p<0.05$).

DISCUSSION

Experiment I demonstrated that most amino acid concentrations peaked at 5 h and returned to near basal level at 24 h after feeding. From the 6 h plasma amino acid concentrations it would appear that post-absorptive concentrations might have been reached long before 24 h. In mammals, including rats (Swendseid et al., 1963; Young and Zamora, 1968), pigs (Puchal et al., 1962), dogs (Longenecker and Hause, 1959) and humans (Young and Scrimshaw, 1970) severe essential amino acid deficiency causes a decrease in the limiting amino acid during the absorptive phase, with a return toward normal 12-24 h after meal. Murai et al. (1987) and Schuhmacher et al. (1997) reported similar results that the plasma concentrations of arginine, leucine, isoleucine, valine, phenylalanine and threonine from fish force-fed crystalline amino acids at 1% body weight (dry-matter) peaked at 6-9 h and returned to baseline by 24-32 h post feeding in rainbow trout.

Post-absorptive (24 h after feeding) plasma free arginine concentrations (PAarg) of the trout increased as dietary protein increased, with somewhat of a plateau occurring at about 1.38% of dietary arginine. Perhaps this is the concentration of dietary arginine that results in "regulation" (either reutilization for protein synthesis or oxidation of excess of that mobilized) of body arginine during the post-absorptive phase.

Table 6. 0 h and 24 h post feeding plasma free arginine concentrations (nmol/ml) from fish fed the various levels of dietary arginine (Experiment I, II and III)[1]

	0 h post feeding value	24 h post feeding value
Exp. I	79±11 (1.12%)[2]	131±13 (2.28%)[3]
Exp. II		66±5 (0.48%)
		132±15 (2.58%)
Exp. III	143±27 (2.28%)[4]	87±18 (0.48%)
		135±33 (2.58%)

[1] Values are means±SD from four fish where the means in each row with different superscripts are significantly different ($p<0.05$).

[2] 0 h post feeding value is 24 h post feeding value from fish fed commercial diet containing 1.21% arginine.

[3] 24 h post feeding value from fish fed experimental diet (dietary arginine levels).

[4] 0 h post feeding value is 24 h post feeding value from fish fed basal diet containing 2.28% arginine.

In Experiment II the effects of alterations of dietary arginine intake on post-prandial (5 h after feeding) plasma free arginine concentrations (PParg) were dependent upon the relative adequacy of the dietary arginine supply. PParg from fish fed 0.48 and 1.08% arginine diets were not significantly different; however, PParg increased with increasing dietary arginine from 1.08 to 2.58%. In the chicks, Zimmerman and Scott (1967) found that the dose-response curves for lysine, valine and arginine in the plasma remained almost flat initially and then increased at the point when the intake of each amino acid just exceeded the level required for maximum growth. In rats, McLaughlan and Illman (1967) found that the dietary level of each essential amino acid that supported the concentration of the amino acids after overnight food deprivation, was the same as that published for the requirements for each amino acid.

If the breakpoint was taken as the requirement of arginine for the trout in the present experiment, the requirement would be 1.03% of diet on the basis of PParg, considerably lower than that shown for the dose-response curve using maximum growth (Ogino, 1980; Walton et al., 1986). PAarg increased with dietary arginine from 0.48% to 1.38%, then showed a breakpoint with a slight continued slope. If this breakpoint were used as the arginine requirement the arginine requirement of trout would be 1.38% dietary arginine. The latter breakpoint is close to the requirement as determined by Ogino (1980) who reported that the arginine requirement of rainbow trout was 1.4% of diet. Kim et al. (1992) estimated the arginine requirement of rainbow trout as 1.41% of diet based on the growth data when L-amino acid mixture, casein and gelatin were used as protein source. Other reported estimates of the arginine requirement of trout ranged between 1.2-1.8% of the diet (Kaushik, 1979; Walton et al., 1986). Since the breakpoint of post-prandial plasma essential amino acids has not been consistently found at the requirement for all essential amino

acids in other species, more work needs to be done before the breakpoint of post-prandial plasma essential amino acids should be taken as the requirement in trout or other fish.

Experiment II showed that PParg (157±22 nmol/ml) was higher than PAarg (66±22 nmol/ml) for fish fed 0.48% arginine diet (less than half of the estimated requirement). This response indicates a basic difference between rainbow trout and mammals and birds in the metabolic response to a dietary deficiency of arginine. Perhaps arginine is catabolized more slowly and thus is available for protein synthesis and gluconeogenesis over a longer period of time after a given meal in rainbow trout. This is not true for all amino acids in fish since for methionine in sea bass (Thebault, 1985) and lysine in rainbow trout (Schuhmacher et al., 1997) dietary deficiencies of these amino acids cause a decrease in their concentrations in post-prandial plasma.

Experiment III confirmed the results from Experiment II that PParg from fish fed the arginine deficient diet were higher than PAarg from fish fed either the arginine deficient diet (0.48%) or the arginine adequate diets (1.68-2.58%). This experiment shows that the response of PAarg pattern in trout is not similar to those of mammals and birds. PAarg from fish fed the arginine deficient diet was lower than that from fish fed the arginine adequate diet (Table 5). This might indicate that PAarg concentrations are dependent upon the previous arginine intake.

In conclusion, these results show that post-prandial plasma arginine concentrations are responsive to dietary arginine level. Feeding a diet severely deficient in arginine to trout results in a postprandial rise, not fall in plasma arginine concentration, in contrast to decreases found in birds and mammals. Breakpoint analysis using PParg resulted in a breakpoint at 1.03% dietary arginine, whereas using PAarg it resulted in a breakpoint at 1.38% dietary arginine. However, validity of using these breakpoints to estimate arginine requirement needs further study.

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A mixture of cottonseed meal, soybean meal and animal byproduct mixture as a fish meal substitute: growth and tissue gossypol enantiomer in juvenile rainbow trout (*Oncorhynchus mykiss*)

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Summary

Diets incorporating three different sources of extracted cottonseed meal (CM), soybean meal and an animal protein mixture were evaluated for juvenile rainbow trout. Fish averaging 0.96 g were divided into groups of 30; 3 groups per treatment, and each group was fed one of four diets for a 16-week period. Fish meal (FM) was replaced on a 25% protein basis by each of three different sources of CM from California (CA), Tennessee (TN), and Arkansas (AR), U.S.A. In the three CM-containing diets another 25% soybean meal protein and 50% animal protein mixture were also incorporated to completely replace FM protein. The results of growth rate and feed utilization showed that FM could be entirely replaced by a mixture of plant proteins (CM and soybean meal) and animal by-product proteins. Hematocrit levels were significantly lower in the group fed CM-containing diets than in the control. The findings suggest that CM can be used as a good protein source by the incorporation of at least 15% in diets (25% of fish meal protein replacement), and that the nutritive values of CM in juvenile trout can be different due to their different origin. Significantly higher concentrations of total gossypol were found in faeces of CM-TN (5.8 ± 0.4 μmol/g) and CM-AR (5.6 ± 0.6) groups than in that of CM-CA (3.7 ± 0.4) group. It was documented that gossypol enantiomers, present in an equal proportion in diets, selectively accumulated in liver and bile, whereas equal proportions of (+)- and (−)-enantiomers were found in whole-body and faeces. Depending on CM source, fish can absorb approximately 35–50% of dietary gossypol, and the majority of the absorbed gossypol seemed to be excreted.

Introduction

Feedstuffs of animal origin are generally considered to be of higher quality than those of plant origin, primarily because of their higher protein content and superior complement of indispensable amino acids (Robinson and Li 1998). In recent years there have been efforts to increase the amount of ingredients of plant origin and studies have reported some success in replacement of fish meal (FM) in diets for large rainbow trout, *Oncorhynchus mykiss*, using soybean meals and protein concentrates (Kaushik et al. 1995; Moyano et al. 1992), soybean and corn gluten meal mixtures (Gomes et al. 1995), and a combination of several alternative protein sources (Yamamoto et al. 1995). Soybean meal was the most frequently studied dietary ingredient as a FM replacement in diets for many fish. Animal by-products, such as poultry by-product meal, meat and bone meal, feather meal, and blood meal, have

also been incorporated in practical fish feeds (MURAI 1992), and have individually been used as an animal protein source for FM replacement (DAVIES et al., 1990; FOWLER 1991; HIGGS et al., 1979; LUZIER et al., 1995). However, when compared to FM-based control diets, diets free of fish meal resulted in general inferior growth in salmonids, (MAMBRINI et al., 1999). By-products of cottonseed are used in diets for both terrestrial animals (COLIN-NEGRETE et al. 1996) and fish (HENDRICKS et al., 1980) because of its high protein content. Cottonseed meal (CM) has been examined in diets of fish such as channel catfish, *Ictalurus punctatus* (DORSA et al., 1982; ROBINSON and BRENT 1989; ROBINSON and LI 1994; ROBINSON and TIERSCH 1995), rainbow trout (HENDRICKS et al., 1980; HERMAN 1970; ROEHM et al., 1967), and tilapia, *Oreochromis niloticus* (EL-SAYED 1990; ROBINSON et al., 1984). Despite its high nutritional value, cottonseed contains gossypol, a polyphenolic compound, which is toxic to fish (HERMAN 1970; RINCHARD et al., 2000) and terrestrial animals (COLIN-NEGRETE et al., 1996; MAKINDE et al., 1997). In most studies, gossypol concentrations in fish tissue were either not analysed or were analysed by a colorimetric method with anisidine (CHAMKASEM 1988; FISHER et al., 1987), which can overestimate gossypol. Data on utilization of CM in fish diets in combination with animal by-product and the resulting gossypol accumulation and/or excretion are not available. There are wide variations in the ratio of gossypol enantiomers present in different species of cotton plants (*Gossypium* species) and even within different tissues in the same plant (CASS et al., 1991; JAROSZEWSKI et al., 1992). However, only few authors reported the selective accumulation of enantiomers in tissues of animals ingesting gossypol (KIM et al., 1996), and only one report exists on catfish (ROBINSON and TIERSCH 1995). We, therefore, evaluated three different sources of solvent extracted CM containing an equal proportion of gossypol isomers, as a FM substitute, incorporated in combination with soybean meal and an animal protein mixture in diets for juvenile rainbow trout. Diets were evaluated by fish growth rate, feed utilization, gossypol 'absorption' and concentrations in tissues, and the digestibility and concentrations of protein and minerals (phosphorus and iron) in fish body and faeces. For the first time concentrations of separate gossypol isomers in tissues and faeces of fish were analysed using specific high performance liquid chromatography (HPLC) methods employing simultaneously both UV and electrochemical detectors.

Materials and methods

Diets

Four experimental diets were formulated to be isonitrogenous and isocaloric in terms of crude protein (47%) and gross energy (17.5 MJ/kg) (Table 1). The energy value of each diet was estimated on the basis of mammalian physiological fuel values, i.e. 16.7 KJ/g protein or carbohydrate and 37.7 KJ/g lipid (LEE and PUTNAM 1973). Dietary FM protein was substituted with 50 or 100% animal protein mixture (APM) and the diets marked as APM50 and APM100, respectively. The APM consisted of equal amounts of meat and bone meal (50% protein, 8.5% lipid), blood meal (92% protein, 0.3% lipid), poultry by-product meal (58% protein, 14% lipid) and feather meal (85% protein, 2.5% lipid). For CM-containing diets, FM was replaced on a 25% protein basis by one of three different sources of CM from California (CA), Tennessee (TN), and Arkansas (AR), USA. These diets are referred to as CM-CA, CM-TN, and CM-AR, respectively. In the CM-containing diets the remaining protein consisted of 25% soybean meal (SM) and 50% APM. The three different sources of CM were solvent extracted meals and the total gossypol concentrations were 1.07, 1.65, and 1.53%, for the CM from California, Tennessee, and Arkansas, respectively. The proportions of (+)- and (–)-isomers of the CM were 53 : 47, 54 : 46, and 53 : 47, respectively. The analysed concentrations of dietary total gossypol were 0.11, 0.16, and 0.16% for the CM-CA, CM-TN, and CM-AR, respectively. Experimental diets were

Table 1. **Composition of the experimental diets (% of dry matter)**

Experimental diets	Control	APM50	APM100	CM-CA	CM-TN	CM-AR
Ingredients[1]						
Fish meal, menhaden	20.00	10.00	–	–	–	–
Fish meal, herring	20.00	10.00	–	–	–	–
Animal Protein Mixture[2]	0.00	19.84	39.67	19.84	19.84	19.84
Cottonseed meal-CA[3]	–	–	–	15.66	–	–
Cottonseed meal-TN[3]	–	–	–	–	14.71	–
Cottonseed meal-AR[3]	–	–	–	–	–	15.97
Soybean meal	–	–	–	14.72	14.72	14.72
Krill meal (hydrolysate)	5.00	5.00	5.00	5.00	5.00	5.00
Wheat middling	28.00	27.00	26.00	11.80	13.00	11.40
Corn gluten meal	11.60	11.80	12.00	15.30	15.00	15.40
Yeast (brewer)	6.00	6.00	6.00	6.00	6.00	6.00
Vitamin mixture[4]	0.50	0.50	0.50	0.50	0.50	0.50
Mineral mixture[5]	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin C[6]	0.05	0.05	0.05	0.05	0.05	0.05
Choline chloride	0.10	0.10	0.10	0.10	0.10	0.10
Menhaden fish oil	8.00	8.90	9.80	10.50	10.50	10.50
Cellulose	0.25	0.31	0.38	0.03	0.08	0.02
Proximate analyses						
Crude protein	46.8	47.2	48.3	48.2	48.3	47.4
Crude Lipid[7]	14.4	14.4	14.3	14.0	14.0	14.0
Gross energy (MJ/kg)[7]	17.5	17.5	17.5	17.5	17.5	17.5

[1] The ingredients were purchased from: fish meal (herring), Ampro Fisheries Co. Reedville, Virginia, USA; fish meal (menhaden), Baker Co., Stamford, Connecticut, USA; soybean meal, Archer Damets Midland Co., Fostoria, Ohio, USA; Krill meal, Specialty Marine Products Ltd, Vancouver, Canada; wheat middling, ADM Co., Loudonville, Ohio, USA; corngluten meal, Baker Trading Co., Dayton, Ohio, USA; Yeast (brewer), Alltech, Chicago, Illinois, USA; mineral mixture (Bernhart Tomarelli salt mixture), choline chloride, and cellulose, ICN Pharmaceuticals Inc., Costa Mesa, California, USA; fish oil (menhaden), Cereal By-products Co., Chicago, Illinois, USA

[2] Animal protein mixture was composed of equal amounts of blood meal (American Protein Co., Ames, IA), meat & bone meal (Inland Products Co., Clyde, OH), feather meal (American Protein Co., Ames, IA), and poultry by-product meal (Holmes By-products Co., Millorsburg, OH). Its crude protein and lipid levels were 68.25% and 6.5%, respectively

[3] CA, California source; TN, Tennessee source; AR, Arkansas source

[4] Roche Performance Premix composition per g of the vitamin mixture: vitamin A, 2646 IU; vitamin D3, 221 IU; vitamin E, 66.1 IU; vitamin B12, 13 µg; riboflavin, 13.2 mg; niacin, 61.7 mg; d-pantothenic acid, 22.1 mg; menadione, 1.32 mg; folic acid, 1.76 mg; pyridoxine, 4.42 mg; thiamin, 7.95 mg; d-biotin, 0.31 mg. Hoffman-La Roche, Inc., Nutley, NJ

[5] Five mg Se in the form of sodium selenite per kg; Bernhart Tomarelli salt mixture (ICN Pharmaceuticals Inc., Costa Mesa, CA)

[6] Phospitan C (Mg-L-ascorbyl-2-Phosphate), Showa Denko K. K. Tokyo, Japan

[7] Calculated based on compositions of the ingredients used (NRC 1993)

cold-pelleted into 2.0 mm diameter size, freeze-dried to approximately 5% moisture, crushed into desirable particle size (0.4–2.0 mm), and stored at −20 °C until use.

Fish, facility and feeding trial

The feeding trial was performed at the Piketon Research and Extension Center aquaculture facility with juvenile rainbow trout (London, Ohio, registered strain) averaging 0.96 ± 0.07 g initial weight. Prior to the feeding trial fish were fed a commercial diet (Bioproducts, Inc., Warrington, OR) for two weeks to allow for adjustment to the experimental conditions. Fish were randomly distributed into groups of 30; 3 groups per treatment. Each

experimental diet was fed to triplicate groups of fish with the feeding rates ranging from 4% of fish weight at the beginning to 2% at the end of the feeding trial (NRC 1993). All procedures and handling of animals were conducted in compliance with the guidelines of the Institutional Laboratory Animal Care and Use Committee, The Ohio State University. The fish were fed three times per day, 7 days a week, for 16 weeks. The feeding trial was conducted in 50 l flow-through circular fibreglass tanks, supplied with well water at a flow rate of 1.8–2.0 l/min. Supplemental aeration was also provided to maintain dissolved oxygen levels near saturation. Water temperature increased gradually from 8 to 15 °C during the experiment and a diurnal light:dark cycle was regulated at 12 h:12 h. Total fish weight in each tank was determined every 4 weeks to check their growth and to adjust the feeding rate. Feeding was stopped 24 h prior to weighing.

Sample collection and analysis

Analyses of crude protein, moisture and ash were performed by standard procedures (AOAC 1995). At the end of the feeding trial all fish were weighed and counted to calculate percent body weight gain (PWG; body wt gain × 100/initial body wt), feed conversion (FC; body wt gain/dry feed consumed), protein efficiency ratio (PER; body wt gain/protein intake), specific growth rate (SGR; [ln final body wt − ln initial body wt] × 100/days), and survival. Hematocrit was determined by the microhematocrit method (Brown 1980) on three fish randomly selected per group (total 9 fish per treatment). For histological examination, livers from three randomly selected fish in each dietary replicate were used. Tissues were preserved in 10% neutral buffered formalin, dehydrated through a graded series of alcohol, and embedded in paraffin. Sections were cut at 3–4 μm, mounted on glass slides and stained routinely with hematoxylin and eosin followed by clearing through xylene and cover slipped over Permount medium.

For gossypol analysis, two fish were randomly selected from each dietary group (total 6 fish per treatment) and killed to collect the liver and bile samples. Three fish per each dietary group (total 9 fish per treatment) were killed for the whole-body analysis of gossypol. For gossypol analysis, faeces were collected after 16 weeks of feeding. Mineral compositions of diets and whole-body was determined by the inductively coupled plasma (ICP) emission spectrophotometric method with the use of ARI-3560 Spectrometer (Applied Research Laboratories, Valencie, CA) according to Watson and Isaac (1990).

Faeces collection and apparent digestibility test

The indirect method described by Cho and Kaushik (1990) was used to calculate the apparent 'digestibility' coefficient (ADC), with chromic oxide (0.5 g per 100 g feed on dry matter basis) as the inert indicator. The apparent 'digestibility' coefficient of protein and gossypol 'absorption' was calculated using the following formula:

$$ADC_{nutrient} = [1 - (NF/ND \times CrD/CrF)] \times 100$$

where: NF = % nutrient in faeces, ND = % nutrient in diet, CrD = % chromic oxide in diet, and CrF = % chromic oxide in faeces. Faeces were collected with a modified faecal collection system (Yamamoto et al., 1998). From the 12th week of feeding trial, fish were fed with the 0.5% chromic oxide-containing diets to facilitate the apparent 'digestibility' test until the end of the feeding trial. After 16 weeks of feeding, all fish of each treatment (three groups) were transferred to three 50 L collection tanks having a steep conical bottom connected to a faeces collecting chamber. Water flowed to the top and out at the bottom of the conical tanks at a rate of less than 0.4 l/min. Fish were prevented from stirring the faeces by a circular net placed at the base of the tank. To collect faeces, all the fish were fed their respective diets containing 0.5% chromic oxide to satiation each morning at 08:00, and again at 11:00. The faeces were collected every 40 min for 7 h, and immediately frozen at −20 °C.

The fish were fed again their respective diets at 20:00 on the same day, collection tanks cleaned, and the faeces were collected the next day at 08:00. The collected faeces were immediately frozen at −20 °C and then stored at −80 °C until analysis.

Analysis of gossypol

Gossypol in diets, liver, bile, whole-body, and faeces were determined by HPLC according to the method described by KIM and CALHOUN (1995), with some modifications. Wet liver tissue was used for analysis of (+)- and (−)-enantiomers of gossypol because preliminary assays revealed that the gossypol concentration in freeze-dried liver samples were lower than that from wet samples. The preliminary assay was conducted in triplicate to compare the two processing methods. The wet liver and freeze dried diets, whole-body, and faeces were weighed, and 5–10 volumes of complexing reagent added to obtain the 2-amino-1-propanol derivatives of (+)- and (−)-enantiomers of gossypol. The complexing reagent was composed of 2 ml 2-amino-1-propanol (Sigma Chemical, St. Louis, MO), 10 ml glacial acetic acid (Sigma Chemical) and 88 ml N, N-dimethylformamide (Sigma Chemical). The samples were homogenized in complexing reagent on ice for 10–40 s, heated at 95 °C for 30 min, cooled on ice, and then centrifuged at 1500 × *g* for 5 min. For determination of gossypol from bile, the homogenization step was omitted. After centrifugation, an aliquot of the supernatant was diluted with mobile phase to obtain a desirable concentration, centrifuged again at 1500 × *g* for 5 min, and filtered through a syringe filter (0.45 μm, Whatman Inc., Clifton, NJ) before injection to HPLC.

The HPLC system consisted of a Beckman 506 A solvent delivery system equipped with a 20 μl injection loop connected to a 4.6-mm × 150 mm Shodex C-18 column (Showa-Denko, Shoko Co. Ltd, Tokyo, Japan) packed with an octadecyl-bonded porous silica gel (5 μm), and both a UV detector (Programmable detector module 166, Beckman Instruments Inc., San Ramon, CA) set at 254 nm and an electrochemical detector (Model LC-4C; BAS, West Lafayette, IN) set applied potential at 0.75 V. The mobile phase was made of 80 ml acetonitrile and 2 mM KH_2PO_4 (final concentration) dissolved in 100 ml distilled water (HPLC grade) adjusted to pH 3.0 with H_3PO_4. Standards of (+)- and (−)-enantiomers of gossypol were provided by Dr Quezia B. Cass, Departamento de Química, Universidade Federal de São Carlos, São Carlos, Brazil (CASS et al., 1999). The retention time for (+)- and (−)-enantiomers of gossypol were 2.1 and 3.4 min, respectively, with a flow rate of 1.8 ml/min. Recovery rates were higher than 92% for both gossypol enantiomers and the detection level was 1 ng/20 μl of injection volume with a signal-to-noise ratio of 3.

Statistical analysis

Each experimental diet was fed to three groups of fish by a completely randomized design. Differences among dietary treatments were tested by one-way ANOVA, and means were compared using Tukey's multiple comparison test by the SPSS statistical package (Version 9.0, SPSS Inc., Chicago, IL). The percentage data of weight gain, specific growth rate, and hematocrit were arcsine transformed before the ANOVA analysis. Differences were considered significant at $p < 0.05$.

Results

No significant differences in fish body weight were found among all groups until week 8, but fish began to show differences in growth rates from the 12th week, followed by significant differences at the 16th week (Fig. 1). Final body weight gains and feed conversion by fish during the 16-week feeding trial are shown in Table 2. Body weight gain, feed conversion and specific growth rate of fish fed CM-TN and CM-AR diets were not significantly different compared to those of fish fed the FM-based control diet. The

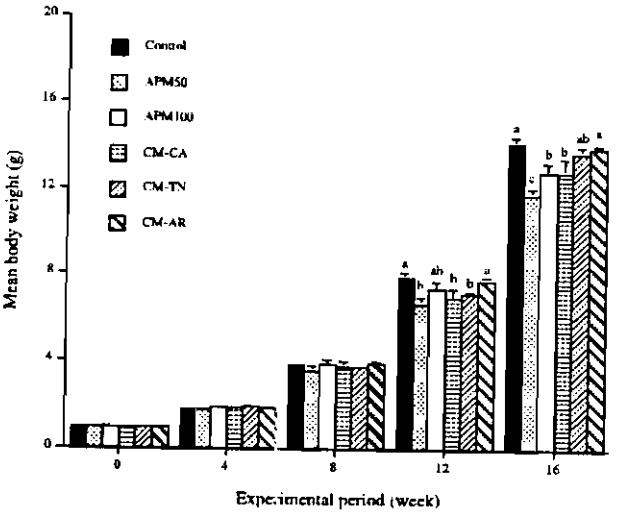

Fig. 1. Changes in mean body weight of rainbow trout fed the experimental diets for 16 weeks. Values are means ± SD of triplicate groups. Different letters (a, b) indicate significantly different values ($p < 0.05$)

Table 2. **Percentage body weight gain (BWG), feed conversion (FC), protein efficiency ratio (PER), specific growth rate (SGR) and hematocrit of juvenile rainbow trout fed experimental diets for 16 weeks[1]**

Diets	BWG (%)	FC	PER	SGR (%)	Hematocrit (%)
Control	1370 ± 17.00 ^{a}	1.03 ± 0.01 ^{a}	2.28 ± 0.02 ^{a}	2.40 ± 0.01 ^{a}	44 ± 1.10 ^{a}
APM50	1129 ± 14.21 ^{c}	0.87 ± 0.00 ^{c}	1.86 ± 0.00 ^{c}	2.24 ± 0.01 ^{c}	40 ± 0.73 ^{b}
APM100	1235 ± 27.23 ^{bc}	0.92 ± 0.02 ^{bc}	1.94 ± 0.04 ^{bc}	2.31 ± 0.02 ^{b}	36 ± 1.52 ^{c}
CM-CA	1235 ± 39.01 ^{bc}	0.91 ± 0.03 ^{bc}	1.92 ± 0.06 ^{bc}	2.31 ± 0.03 ^{b}	39 ± 1.04 ^{b}
CM-TN	1330 ± 16.17 ^{ab}	0.96 ± 0.01 ^{ab}	2.03 ± 0.02 ^{b}	2.37 ± 0.01 ^{ab}	39 ± 0.94 ^{b}
CM-AR	1350 ± 10.93 ^{a}	1.00 ± 0.01 ^{a}	2.18 ± 0.01 ^{a}	2.39 ± 0.01 ^{a}	39 ± 1.09 ^{bc}

[1]Means of triplicate groups; Values ± SD in the same column with different superscript are significantly different ($p < 0.05$)

protein efficiency ratio of fish fed the CM-AR diet was also not significantly different compared to that of fish fed the control diet. However, fish fed APM50, APM100, and CM-CA diets exhibited significantly lower growth performances than the fish fed the control diet. A significant decrease in hematocrit was found in fish fed diets containing CM and/or APM compared to fish fed the control diet. No differences were observed in whole-body protein and ash concentrations among all the groups (results not presented). No mortality was observed and differences in the palatability of the diets were not noticed during the 16 weeks of the feeding trial.

The ratio of (+)- and (−)-enantiomers was equal in the three CM-containing diets. Gossypol concentrations in liver, bile, whole-body, and faeces are presented in Fig. 2. No differences in gossypol concentrations were found among treatments in liver, bile and whole-body of the fish. Significantly higher concentrations of total gossypol were found in faeces of CM-TN (5.8 ± 0.4 μmol/g) and CM-AR (5.6 ± 0.6) groups than in that of CM-CA (3.7 ± 0.4) group. This trend in total gosssypol was also observed in both (+)- and

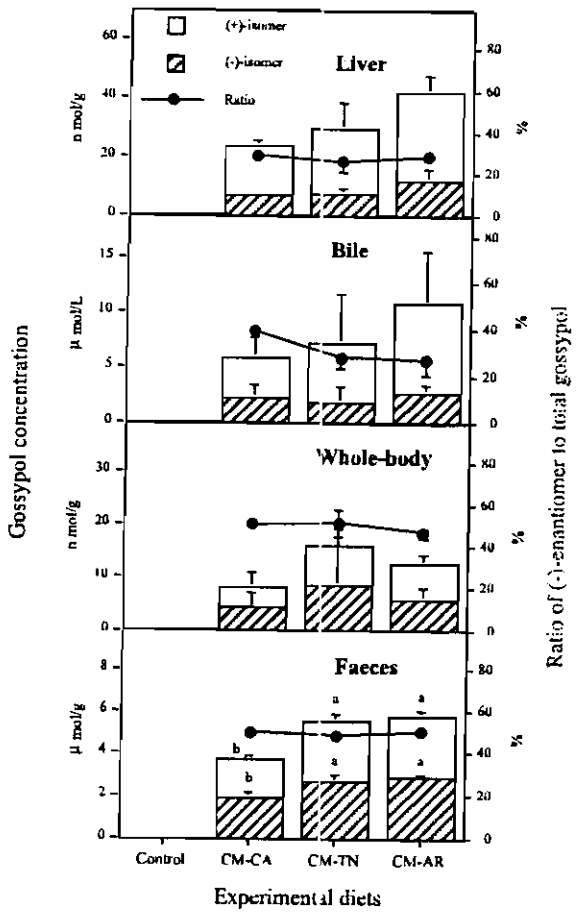

Fig. 2. Concentrations of gossypol enantiomer in liver, bile, whole-body, and excreted faeces of juvenile rainbow trout fed the control (fish meal-based) and test diets containing three different sources of cottonseed meal during 16 weeks. Values are means ± SD of triplicate groups. Different letters indicate significantly different values ($p < 0.05$)

(−)-gossypol enantiomers. A significant correlation ($r^2 = 0.85, p < 0.001$) was found between gossypol concentrations in diet and faeces. Gossypol was not detected in tissues or faeces of the fish fed the control diet. Interestingly, the ratio of (−)-enantiomers to total gossypol differed, depending on tissues or faeces. In liver and bile, the percentage ratios of (−)-enantiomers to total gossypol were less than 30% which means that over 70% of (+)-enantiomer was selectively retained in tissue. However, an approximately equal proportion of each (+)- and (−)-enantiomer were found in the whole-body and faeces of fish. No significant histopathological changes were found in liver tissues of fish examined. The colour of livers were not different between fish fed CM-containing and control diets, however, yellowish liver due to the gossypol deposition was found in tilapia, *Oreochromis* spp. fed the same amount of CM for 16 weeks in our previous study (Mbahinzireki et al., 2001).

Phosphorus and iron concentrations in diets, whole body and faeces are shown in Fig. 3. Phosphorus in faeces of the control group was significantly higher than those of other groups, and iron concentration in faeces was significantly higher only in CM-TN group

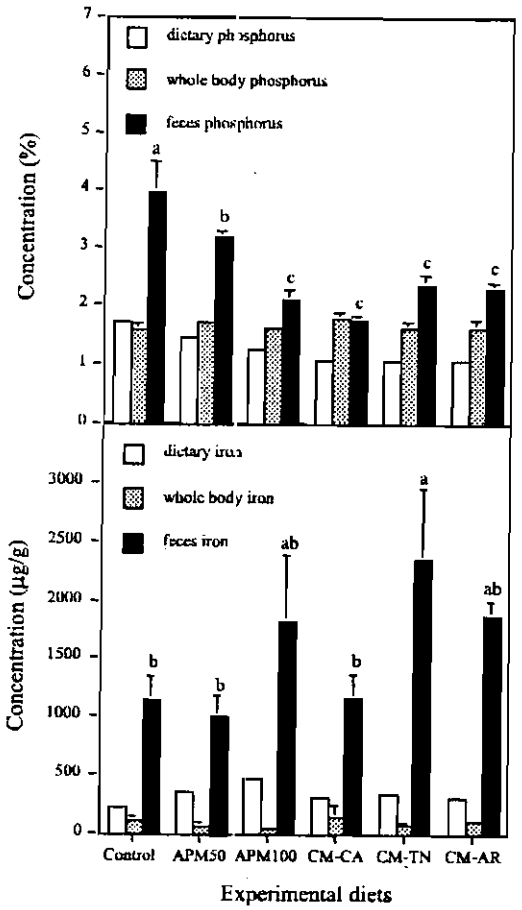

Fig. 3. Concentrations of phosphorus and iron in experimental diets, whole-body, and faeces. Values of whole-body and faeces are means ± SD of triplicate groups. Different letters are significantly different ($p < 0.05$)

compared to the control, APM50 and CM-CA groups. No significant differences were observed in whole body concentrations of phosphorus and iron among treatments. The apparent 'digestibility' of dietary protein and 'absorption' of phosphorus and gossypol after 16 weeks of feeding were different among treatments (Fig. 4). Protein 'digestibility' of diet CM-AR (89.6 ± 1.29%) was significantly higher than those of the control (85.0 ± 1.88%), APM50 (83.4 ± 0.41%), APM100 (80.6 ± 2.10%) and CM-CA (83.5 ± 1.34%) diets. The phosphorus 'absorption' was higher in diets containing CM and/or APM than in the control. The 'absorption' rate of gossypol ranged from 35 to 50% depending on the CM source, and was significantly higher in CM-TN and CM-AR groups than in CM-CA group.

Discussion

The results of the present study are significant because, to our knowledge, it is the first fish meal free and high dietary protein (45%) formulation for juvenile rainbow trout that resulted in comparable growth rate to the FM-based control diet. Furthermore, the fish fed

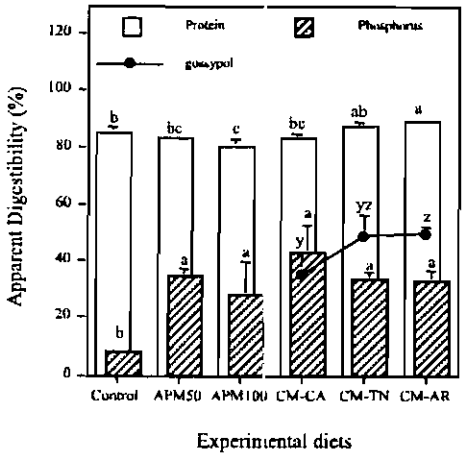

Fig. 4. Apparent 'digestibility' of dietary protein and 'absorption' of phosphorus and gossypol examined after 16 weeks of feeding trial. All the fish from the feeding trial were used for the 'digestibility' test using inert indicator, 0.5% chromic oxide. Values are means ± SD of triplicate groups. Different letters indicate significantly different values ($p < 0.05$)

the FM-based control diet (herring and menhaden FM, 1 : 1) in the present study grew at a rate comparable or higher specific growth rates (2.40 ± 0.01% per day) than indicated earlier in other studies. For instance, SKONBERG et al., (1997) used a similar size of rainbow trout and SGR of fish fed a control diet (herring FM) amounted to 1.42 and 2.56% on restricted or *ad libitum* feeding, respectively, in an 8-week-long study. Rainbow trout weighing 3 g fed a menhaden FM-based diet (*ad libitum*) showed a SGR of 2.77% after 13 weeks of feeding (WATANABE et al., 1993). The feeding period in our study was longer compared to other FM replacement studies that were conducted with juvenile rainbow trout (STICKNEY et al., 1996; YAMAMOTO et al., 1995).

HERMAN (1970) reported that in rainbow trout, growth depression did not occur until dietary free gossypol concentrations were higher than 290 mg/kg. ROBINSON and TIERSCH (1995) found no effect of 200 mg free gossypol per kg diet in channel catfish on growth, feed consumption, feed conversion ratio, and survival. Free gossypol is defined as 'acetone soluble gossypol', whereas bound gossypol can be estimated by subtracting the portion of free form from total gossypol. In the present study the free gossypol concentrations of the diets CM-CA, CM-TN, and CM-AR were 60, 150, and 225 mg/kg, respectively, based on individual CM analysis. The reason for the lower growth rate in CM-CA diet group can be attributed to the inferior nutritive value of CM rather than its gossypol concentration. The nutritive values of CM can differ among different species of cottonseeds processed (CASS et al., 1991; JAROSZEWSKI et al., 1992) and different processing methods (CHERRY et al., 1978; FORSTER and CALHOUN 1995).

Lower hematocrits were found in the group fed CM-containing diets than in the control in the present study. This result is in agreement with the results in rainbow trout broodstock (DABROWSKI et al., 2000), Nile tilapia (MBAHINZIREKI et al., 2001), swine and rats (SKUTCHES et al., 1973, 1974). The reason for the observed lower hematocrit in groups fed CM-containing diets in the present study could be the cumulative effect of gossypol and/or decreased availability of iron in CM causing increased erythrocyte fragility (COLIN-NEGRETE et al., 1996; MAKINDE et al., 1997).

ROEHM et al., (1967) reported that in rainbow trout, the liver was the main organ responsible for accumulation of gossypol and that the gossypol elimination took place

with a considerable delay in the liver. In the present study we found the percent ratio of the (–)-enantiomer to total lower in liver (26–29%) and bile (27–39%) than in the whole-body (47–52%) and faeces (49–51%). This result supported the notion that liver is the main organ for the elimination of absorbed gossypol (ROEHM et al., 1967), and was consistent with the result of tissue gossypol concentrations found in rainbow trout broodstock in our laboratory. In our previous study we observed the highest gossypol concentrations in liver compared to plasma, bile, kidney, muscle, stomach and gametes (results not published). The findings reported here may suggest that liver eliminates (–)-enantiomer more actively than the (+)-enantiomer. Several studies have shown that the (–)-enantiomer has the higher biological activities, such as antifertility, antitumour and toxicity than (+)-enantiomer (BENZ et al., 1990; JOSEPH et al., 1986; SHELLEY et al., 1999; TANPHAICHITR et al., 1988). SMITH and CLAWSON (1965) indicated that the primary pathway of gossypol excretion was via the biliary system. Our results of bile gossypol concentrations supported the hypothesis that the primary pathway of gossypol excretion is via biliary system. We found high concentrations of total gossypol in the faeces of rainbow trout (3.7–5.8 μmol/g dry matter). Heifers exposed to 1300 and 2000 mg of gossypol/kg excreted 0.58 and 2.3 μmol of total gossypol per g faeces, respectively (COLIN-NEGRETE et al., 1996). COLIN-NEGRETE et al., (1996) calculated that between 5 and 15% of gossypol was absorbed by the heifers. ABOU-DONIA and LYMAN (1970) reported that in pigs and hens, which are sensitive to gossypol toxicity, the maximum amount of radioactivity absorbed in tissues was 32.9 and 16.8% of the orally administered dose, respectively. The approach we used to determine the 'absorption' rate of gossypol in trout was to compare the gossypol concentration in diets and excreted faeces by using chromic oxide as a indirect method (CHO and KAUSHIK 1990) and to consider the total consumption and accumulation of gossypol in the whole-body throughout the feeding trial. In the present study, we found a large proportion of dietary gossypol (35–49%) absorbed by the fish in comparison to terrestrial animals (Fig. 4). We also calculated that fish consumed on average 13.1 ± 0.26 g of diet during the 16-week period, leading to a total intake of 13.7–21.4 mg of total gossypol per fish depending on the three different sources of CM-containing diets. This would then result in gossypol intake ranging from 1.07 ± 0.05 to 1.54 ± 0.02 mg per g of fish body weight. However, fish retained only 0.8–1.6 μg gossypol per g of fish body wt indicating that the amount of gossypol that was not excreted through faeces is less than 0.2% of dietary gossypol. Therefore, it may be assumed that the majority of the absorbed gossypol is metabolized to other compounds such as gossypolone, gossypolonic acid and demethylated gossic acid as proposed by ABOU-DONIA and DIECKERT (1975). However, we observed very low amounts of gossypolone in liver tissues (identified by internal standard) showing less than 1% of the total gossypol detected by HPLC (data not shown).

The result of the higher protein 'digestibility' (Fig. 4) in the fish fed CM-AR and CM-TN diets may indicate that in rainbow trout the availability of CM is comparable or a little higher than that of fish meal. Other protein sources, such as soybean, corn gluten, and poultry by-product meal had similar protein availabilities to both herring and menhaden fish meal (RICHE and BROWN 1999; SUGIURA et al., 1998). The significantly lower phosphorus 'absorption' (Fig. 4) in the control in comparison to the other groups can be explained by the significantly improved utilization of fish meal phosphorus by plant ingredients (RICHE and BROWN 1999). This result is also in agreement with the observation by SUGIURA et al., (1998) in coho salmon and rainbow trout, where phosphorus 'absorption' was inversely correlated with dietary levels of calcium and with phosphorus itself. The authors reported higher phosphorus availabilities in soybean and corn gluten meal, as well as poultry by-product and feather meal, than in both herring and menhaden fish meal. In the present study the concentrations of calcium and phosphorus in the control diet (1.67 and 1.63 g/100 g, respectively) was 1.5 and 2.3 times higher than those in CM-CA, CM-TN, and CM-AR diets (results not presented). The reason for the higher trend in

the faeces iron concentrations in CM-TN (significant) and CM-AR (not significant) compared to that of the control (Fig. 3) might be attributed to the characteristic of gossypol chelation by iron. Owing to the fact that gossypol readily reacts with iron, it was found that the reaction in the intestine resulted in the formation of an insoluble complex which is egested in the faeces (MUZAFFARUDDIN and SAXENA 1966; SKUTCHES et al., 1974).

In conclusion, this study demonstrates that CM can be used as a valuable protein source by at least 15% incorporation in diets for juvenile rainbow trout. Fish meal, a traditional protein source in fish feed, can also be completely replaced by a mixture of plant protein (CM and soybean meal) and some other animal by-product proteins. Nutritive values of CM in fish can be different depending on their origin and processing. We documented that fish excrete the (−)-enantiomer faster than the (+)-enantiomer of gossypol, and that approximately 35–50% of dietary gossypol is absorbed by fish, whereas the remaining absorbed gossypol seemed to be excreted with urine and/or through gills. This needs further study.

Acknowledgements

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The patterns of plasma free amino acids after force-feeding in rainbow trout *Oncorhynchus mykiss* (Walbaum) with and without dorsal aorta cannulation

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Abstract

Two experiments were conducted to compare the patterns of plasma free amino acid concentrations after force-feeding in rainbow trout *Oncorhynchus mykiss* (Walbaum) with and without dorsal aorta cannulation. In the first experiment, 35 rainbow trout averaging 504 ± 7.8 g (mean ± SD) were divided into seven groups of five fish each. After 48 h starvation, a group of fish was anaesthetized and blood samples were taken at one of the following time periods: 0, 4, 8, 12, 24, 36 and 48 h after feeding. In the second experiment, five dorsal aorta cannulated rainbow trout averaging 511 ± 6.2 g (mean ± SD) were kept in a cage. After 48 h starvation, the fish were anaesthetized and blood samples were taken from the same fish at 0, 4, 8, 12, 24, 36 and 48 h after feeding. In the first experiment, the concentration of all plasma free amino acids except histidine and glycine peaked at 4 h and returned to the basal level 24 h after feeding. In the second experiment, the concentration of all plasma free amino acids except isoleucine, leucine, phenylalanine and tryptophan also peaked at 4 h and returned to the basal level 24 h after feeding. These results showed that the pattern of plasma free amino acid concentrations from fish with and without dorsal aorta cannulation were similar.

Keywords: plasma free amino acids, force feeding, rainbow trout, dorsal aorta cannulation

Introduction

Plasma free amino acid levels were measured to study amino acids metabolism and to evaluate the quality of dietary protein in trout (Nose 1972; Schlisio & Nicolai 1978; Yamada, Simpson, Tanaka & Katayama 1981; Ogata & Arai 1985; Walton & Wilson 1986; Murai, Ogata, Hirasawa, Akiyama & Nose 1987; Cowey & Walton 1988; Ash, McLean & Westcott 1989; Schuhmacher, Goldberg, Schön, Wax & Gropp 1993; Tantikitti & March 1995; Schuhmacher, Wax & Gropp 1997). In these experiments, blood samples were taken from the caudal vein and artery by needles and syringes in the anaesthetized fish randomly captured from the treatment population. The procedures of these experiments have several major limitations. First, the handling associated with the method is stressful. Second, the method does not allow repeated sampling of the same individual fish, thus requiring large numbers of fish and tanks. These limitations resulted in a large individual variation within treatment in the time course of amino acid appearance in the peripheral blood and peak level.

Procedures for dorsal aorta cannulation and repeated sampling of blood in the same resting fish are well established (Soivio, Westman & Nyholm 1972). The dorsal aorta cannulation allows repeated sampling of the same individual fish when studying changes in the levels of nutrients in the blood circulation. Therefore, the purpose of the present

study was to compare the patterns of plasma free amino acid concentrations in force-fed rainbow trout with and without dorsal aorta cannulation.

Materials and methods

Animals and husbandry

Growing rainbow trout averaging 504 ± 7.8 g (experiment I) and 511 ± 6.2 g (experiment II) mean body weight were obtained from Ewhajung trout farm in Sangju, Korea. For both experiments eight net cages (1.3 × 1.3 × 1.3 m) were placed in a flow-through concrete raceway with a water flow of 60 L min^{-1}. Aeration was provided to maintain dissolved oxygen at 7.4 ± 0.7 mg L^{-1}, and water temperature was maintained at 17 ± 0.2 °C.

Dorsal aorta cannulation

Fish were anaesthetized with 200 p.p.m MS-222 (3-aminobenzoic acid ethyl ester methansulphonate; Sigma, St Louis, MO, USA) for 3–5 min, and placed on a V-shape table, and gills were irrigated continuously with 16 °C water containing 100 p.p.m MS-222 during the operation. A 50-cm-long cannula (Clay Adams PE 50 tubing, Parsippany, NJ, USA) with a bubble about 5–6 cm from one end was washed with the heparinized Cortland saline solution (Houston 1990), and a 13-G needle was used to pierce a hole on the right nostrum (ventral side up) for the cannula to come out. A 19-G needle was used to bore a small hole in the roof of the mouth at the mid-line behind the third gill arc at a 30° angle, and a guitar wire (G) was inserted into the PE 50 as a guide. The proper insertion was indicated by a slow blood flow after the wire was withdrawn from the cannula. A 3-cm^3 syringe with a 23-G needle was used to remove air and blood clot, and the cannula was flushed with the heparin solution. The cannula was sutured behind the bubble on the roof of the mouth, led out from the right nostrum, plugged with a colour head pin, and sutured at the dorsal fin.

Experimental design, diet and force-feeding

In the first experiment, 35 rainbow trout were divided into seven groups of five fish each. After 48 h starvation a group of five fish was anaesthetized with 200 p.p.m MS-222 and blood samples were taken at one of the following time periods; 0, 4, 8, 12, 24, 36 and 48 h after force-feeding a basal diet by the stomach intubation method (Schuhmacher *et al.* 1997) at a rate of 1% body weight on a dry matter basis.

Table 1 Composition of the basal diet (% dry matter)[1]

Ingredient	Proportion (%)
Essential amino acids	17.27
Nonessential amino acids	12.36
Casein[2]	5.00
Gelatine[2]	2.00
Dextrin[2]	27.97
Dextrose[2]	5.00
Cellulose[2]	8.20
Fish oil[3]	10.00
Carboxymethyl cellulose[2]	1.00
$Ca(H_2PO_4).2H_2O$	3.00
Choline bitartrate[2]	1.20
Vitamin mixture[4]	3.00
Mineral mixture[5]	4.00

[1]Diets were neutralized with NaOH to give a final pH of 6.6.
[2]United States Biochemical (Cleveland, OH, USA).
[3]Ewha Oil Company (Pusan, Korea).
[4]Vitamin mixture (mg kg^{-1} feed unless indicated otherwise): vitamin A, 3000 IU; vitamin D3, 2400 IU; vitamin E, 120 IU; menadione sodium bisulphate, 6; vitamin B1-HCl, 15; vitamin B2, 30; vitamin B6-HCl, 15; vitamin B12, 0.06; vitamin C, 300; calcium pantothenate, 150; nicotine amide, 150; inositol, 150; d-biotin, 1.5; choline chloride, 3000; pancreatin, 12.5.
[5]Mineral mixture (mg kg^{-1} feed): $MnSO_4$, 320; $ZnSO_4$, 270; $FeSO_4$, 750; $CuSO_4$, 60; $CoSO_4$, 7; $MgSO_4$, 17.25; K_2SO_4, 212.24; NaCl, 51.88; K_2HPO_4, 136.09; $NaSeO_3$, 0.013; KI, 0.15.

In the second experiment five dorsal aorta-cannulated rainbow trout were stocked in one cage. After 48 h starvation, the fish were anaesthetized with 200 p.p.m MS-222 and blood samples were taken from the same five fish repeatedly at 0, 4, 8, 12, 24, 36 and 48 h after force-feeding the basal diet by the stomach intubation method at a rate of 1% body weight on a dry matter basis.

The basal diet was formulated by modifying the procedure of Kim (1997), and the diet containing 29.6% crystalline amino acid mixture plus 5% casein and 2% gelatine. Ingredients and amino acid composition of the basal diet are shown in Tables 1 and 2, respectively. The basal diet mixture without oil was stored at −80 °C until used. Ingredients were mixed with 10% of fish oil and water before intubation. Fish with and without dorsal aorta

Table 2 Amino acid composition of the basal diet (% dry matter)

	From casein and gelatine	From crystalline amino acids	Total[1]
Essential amino acids			
Arginine	0.353	1.924[2]	2.277
Histidine	0.194	0.725	0.919
Isoleucine	0.252	1.674	1.926
Leucine	0.493	2.702	3.195
Lysine[2]	0.502	1.904	2.406
Methionine	0.152	1.030	1.182
Phenylalanine	0.271	1.742	2.013
Threonine	0.221	1.601	1.822
Tryptophan	0.065	0.462	0.527
Valine	0.350	1.999	2.349
Non-essential amino acids			
Alanine	0.345	1.741	2.086
Aspartic acid	0.483	3.280	3.763
Cysteine	0.019	0.172	0.191
Glycine	0.538	0.758	1.296
Glutamic acid	1.298	3.616	4.914
Proline	0.790	0.568	1.358
Serine	0.374	2.398	2.772
Tyrosine	0.270	1.335	1.605

[1]The amino acid profile was simulated with that of 35% whole chicken egg protein (Robinson, Wilson & Poe 1981).

[2]Lysine-HCl was used in crystalline amino acids mixture.

cannulated were anaesthetized with 200 p.p.m MS-222 and fed the basal diet by the stomach intubation method (diet plus 0.4 parts of distilled water per diet) using a 3-cm^3 syringe.

Sample collection and analysis

Fish were anaesthetized with 200 p.p.m MS222, and 300 μL blood was obtained from fish. In the first experiment blood samples were taken from the caudal vein using a heparinized syringe, and in the second experiment blood samples were taken from dorsal aorta using the cannula. Plasma samples were prepared by centrifugation at 3000 *g* for 10 min. For deproteinization, the plasma samples were mixed with 5-sulphosalicylic acid (458 mmol L^{-1}) in the ratio of 4 : 1, cooled on ice for 30 min and recentrifuged. The protein-free supernatant was dissolved in pH 2.2 lithium citrate sample dilution buffer in the ratio of 1 : 1, and the samples were stored at −80 °C until analysis. The plasma free amino acids were separated and quantified using a S433 amino acid analyser (Sykam, Gilching, Germany) using the ninhydrin method.

Statistical analysis

Data were subjected to ANOVA test using Statistix 3.1 (Analytical Software, St Paul, MN, USA). When a significant treatment effect was observed, a Least Significant Difference test was used to compare means. Treatment effects were considered significant at $P < 0.05$.

Results

Plasma free amino acid concentrations from fish without dorsal aorta cannulation are summarized in Table 3. Plasma free arginine, isoleucine, leucine, methionine, phenylalanine, threonine, tryptophan, valine, alanine and glutamic acid concentrations peaked at 4 h, returned to the basal level between 12 and 24 h, and remained constant between 24 and 48 h after feeding. Plasma free histidine concentration decreased at 8 h and peaked at 36 h after feeding. Plasma free glycine concentration decreased between 4 and 8 h, peaked at 12 h and returned to the basal level at 24 h after feeding.

Plasma free amino acid concentrations from fish with dorsal aorta cannulation are summarized in Table 4. Plasma free arginine, histidine, lysine, methionine, threonine, valine and glutamic acid concentrations peaked at 4 h, returned to near basal line level between 8 and 24 h, and remained constant between 24 and 48 h after feeding. Plasma free isoleucine, leucine, phenylalanine and tryptophan concentrations peaked at 4 h, decreased between 8 and 24 h, and remained constant thereafter. Plasma free glycine concentration decreased between 4 and 8 h, peaked at 12 h and returned to the basal level at 24 h after feeding. Plasma free alanine and aspartic acid concentrations peaked at 4 h and returned to the basal level 48 h after feeding.

Discussion

In force-fed rainbow trout with or without dorsal aorta cannulation after 48 h starvation, most of plasma free amino acids peaked approximately 4 h after feeding, and returned to the basal level at 24 h. Murai *et al.* (1987) reported that the plasma free amino acid concentrations from fish force-feeding

Table 3 Plasma free amino acid concentrations (nmol mL^{-1}) from fish without dorsal aorta cannulation after force-feeding the basal diet (experiment I)[1]

	Time (h) after force-feeding							
	0	4	8	12	24	36	48	Pooled SEM
Essential amino acids								
Arginine	$127^{c,o}$	317^{a}	217^{b}	214^{b}	90^{d}	$118^{c,d}$	$108^{c,d}$	17
Histidine	$173^{b,c,d}$	$199^{a,b}$	115^{d}	$137^{c,d}$	$142^{b,c,d}$	250^{a}	$193^{a,b}$	8
Isoleucine	103^{d}	522^{a}	379^{b}	264^{c}	146^{d}	157^{d}	$140^{c,d}$	29
Leucine	164^{d}	684^{a}	492^{b}	346^{c}	183^{d}	132^{d}	198^{d}	37
Lysine	$513^{a,b}$	663^{a}	$486^{b,c}$	$477^{b,c}$	191^{d}	$406^{b,c}$	$330^{c,d}$	27
Methionine	432^{c}	761^{a}	$604^{a,b}$	$505^{b,c}$	393^{c}	383^{c}	$378^{c,d}$	25
Phenylalanine	126^{d}	649^{a}	527^{b}	326^{c}	135^{d}	$212^{c,d}$	147^{d}	41
Threonine	$279^{c,d}$	533^{a}	364^{b}	289^{c}	181^{o}	$223^{c,d,e}$	$275^{c,d,e}$	21
Tryptophan	7^{c}	28^{a}	16^{b}	10^{c}	$12^{b,c}$	8^{c}	7^{c}	3
Valine	$189^{b,c}$	324^{a}	228^{b}	$160^{c,d}$	$126^{d,o}$	97^{o}	87^{o}	16
Non-essential amino acids								
Alanine	584^{d}	928^{a}	752^{b}	627^{c}	528^{d}	454^{e}	527^{d}	31
Aspartic acid	542^{d}	874^{a}	792^{b}	647^{c}	754^{b}	658^{c}	73^{b}	20
Glycine	674^{b}	526^{d}	432^{d}	724^{a}	$637^{b,c}$	593^{c}	427^{e}	21
Glutamic acid	324^{c}	986^{a}	842^{b}	652^{c}	317^{o}	$363^{d,e}$	401^{d}	52
Serine	275^{d}	754^{a}	693^{b}	456^{c}	435^{c}	294^{d}	324^{e}	35
Tyrosine	73^{c}	354^{a}	265^{b}	162^{c}	96^{d}	88^{d}	91^{d}	19

[1]Values are mean of five fish where the mean values in each row with a different superscript are significantly different ($P < 0.05$).

Table 4 Plasma free amino acid concentrations (nmol mL^{-1}) from fish with dorsal aorta cannulation after force-feeding the basal diet (experiment II)[1]

	Time (h) after force-feeding							
	0	4	8	12	24	36	48	Pooled SEM
Essential amino acids								
Arginine	102^{b}	668^{a}	354^{b}	245^{b}	195^{b}	145^{b}	212^{b}	36
Histidine	141^{b}	327^{a}	150^{b}	93^{b}	94^{b}	91^{b}	135^{b}	15
Isoleucine	113^{c}	654^{a}	465^{b}	327^{c}	$273^{c,d}$	216^{d}	175^{d}	33
Leucine	152^{c}	738^{a}	688^{b}	612^{c}	275^{d}	254^{d}	310^{d}	44
Lysine	$637^{b,c}$	1257^{a}	$1019^{a,b}$	$767^{a,b,c}$	$707^{b,c}$	417^{c}	$753^{b,c}$	51
Methionine	373^{b}	945^{a}	545^{b}	582^{b}	377^{b}	288^{c}	372^{b}	42
Phenylalanine	132^{c}	792^{a}	672^{b}	534^{c}	232^{d}	194^{d}	212^{d}	48
Threonine	253^{c}	641^{a}	437^{b}	392^{b}	274^{c}	252^{c}	206^{d}	27
Tryptophan	8^{c}	31^{a}	27^{b}	19^{c}	12^{d}	11^{d}	12^{d}	2
Valine	175^{d}	432^{a}	318^{b}	233^{c}	$195^{c,d}$	154^{e}	176^{d}	19
Non-essential amino acids								
Alanine	567^{c}	1105^{a}	984^{b}	842^{c}	727^{d}	682^{d}	593^{e}	38
Aspartic acid	563^{c}	1087^{a}	872^{b}	784^{c}	$813^{b,c}$	721^{d}	594^{e}	32
Glycine	653^{c}	$582^{c,d}$	472^{d}	899^{a}	724^{b}	632^{c}	474^{d}	28
Glutamic acid	351^{c}	1171^{a}	983^{b}	725^{c}	513^{d}	479^{d}	452^{e}	54
Serine	251^{c}	914^{a}	783^{b}	561^{c}	541^{c}	411^{d}	395^{d}	47
Tyrosine	81^{d}	401^{a}	195^{b}	212^{b}	105^{c}	$93^{c,d}$	87^{d}	23

[1]Values are mean of five fish where the mean values in each row with a different superscript are significantly different ($P < 0.05$).

crystalline amino acids peaked at 6 h, and returned to the basal level at 24 h. A similar result was reported by Schuhmacher *et al.* (1997). Walton and Wilson (1986) hand-fed a complete diet based on casein, and measured peak levels at 12 h. Yamada *et al.* (1981) observed that plasma free amino acid concentrations peaked between 24 and 36 h after force-feeding casein, and 12 h after force-feeding a crystalline amino acid mixture. Walton and Wilson (1986) and Cowey and Walton (1988) suggested that this lag phase was caused by an extended starvation period (7 days) and by stress owing to force-feeding. The lag phase could probably be traced back to other factors influencing amino acid uptake, i.e. dietary (nonprotein) energy source and energy content (Ogino & Takeuchi 1976; Pfeffer 1982).

Force-feeding crystalline amino acid diet leads to a peak of plasma free amino acids earlier than with force-feeding intact protein. Crystalline amino acids favour rapid intestinal amino acid uptake, which in turn leads to a fast supply of the plasma, and also an intensified amino acid catabolism (Schuhmacher *et al.* 1997; Rodehutscord, Borchert, Gregus, Michael, & Pfeffer 2000). In the case of amino acids entering plasma from intact protein, absorption of amino acids continues over a longer period of time and thereby extends the period over which the amino acids are available for protein synthesis (Tantikitti & March 1995). Plakas, Lee, Wolke & Meade (1985) examined plasma free amino acid levels as an index of protein quality in the diet. The bioavailability of lysine in early maillard browned protein was determined by plasma free lysine response in rainbow trout.

Determination of plasma essential amino acid levels could allow accurate prediction of dietary essential amino acid needs in humans (Young, Hussein, Murray & Scrimshaw 1971), chicks (Zimmerman & Scott 1965), rats (McLaughlan & Illman 1967), pigs (Mitchell, Becker, Jensen, Harmon & Norton 1968) and rainbow trout (Kaushik 1979). Different feeding and blood sampling procedures, such as dosage, fish size, tube size, and/or texture of the material, may affect the pattern of plasma free amino acid concentrations and peak occurrence. Yamada *et al.* (1981) and Ng, Hung and Herold (1996) observed that plasma free amino acid concentrations fell 2 h after force-feeding. This may have been caused by the effect of force-feeding. Stress leads to a net catabolism of peripheral proteins, elevation of plasma free amino acid levels and increased amino acid metabolism in several fish species (van der Boon, van den Thillart & Addink 1991; Vijayan, Ballantyne & Leatherland 1991).

The present experiment results show that the patterns of most plasma free amino acid concentrations from force-fed rainbow trout with and without dorsal aorta cannulation were similar, and the dorsal aorta cannulation did not affect the pattern of plasma free amino acid concentrations in rainbow trout. These result indicate that force-fed rainbow trout might recover from the stress of dorsal aorta cannulation within 48 h of the operation, and the dorsal aorta cannulation would allow repeated sampling on the same individual fish to study nutrient metabolism in the blood circulation. This is in line with findings in rainbow trout (Brown, Eales & Hara 1986) and channel catfish *Ictalurus punctatus* (Ratinesque) (Mazik, Plakas & Stehly 1994) subjected to dorsal aorta cannulation. The pattern of plasma free amino acid concentrations from force-fed rainbow trout with dorsal aorta cannulation may be useful in determining the optimum blood sampling time and evaluating protein quality and essential amino acid requirements. However, concentrations of most plasma free essential amino acids in rainbow trout with dorsal aorta cannulation were higher than those without dorsal aorta cannulation. This may have been caused by the stress effects of repeated blood samplings. Thus, further studies should be done to refine the optimum blood sampling time and to confirm the stress effects of anaesthetization in rainbow trout with dorsal aorta cannulation.

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Replacement of Fish Meal by a Mixture of Animal By-Products in Juvenile Rainbow Trout Diets

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Abstract.—A mixture of animal by-products (MAB) was tested to replace fish meal (FM) in diets for juvenile rainbow trout *Oncorhynchus mykiss*. Fish averaging (±SD) 0.96 ± 0.07 g were divided into 15 groups, and 3 groups were fed one of five isonitrogenous diets replacing 0, 20, 40, 60, or 100% of FM protein with similar percentages of MAB (control, MAB20, MAB40, MAB60, or MAB100, respectively). The MAB consisted of 25% meat and bone meal, 24.5% leather meal, 20% squid liver powder, 15% feather meal, 7.5% blood meal (spray-dried), 7.5% poultry by-product meal, and 0.25% each methionine and lysine. After 16 weeks of feeding, fish fed diets MAB40, MAB60, and MAB100 exhibited significantly lower growth performance than the fish fed the control and MAB20 diets. Apparent digestibility estimates of protein with different collection times (every 40 min and after 12 and 48 h) did not show, in a feces collection system, protein leaching into the water for up to 48 h. The MAB substitution of up to 60% of FM protein in diets did not show differences in apparent protein digestibility (85.8 ± 1.05% for MAB20, 83.1 ± 0.45% for MAB40, and 82.7 ± 2.67% for MAB60) compared with the control (85.0 ± 1.88%), whereas in the MAB100 group digestibility (77.7 ± 4.42%) was significantly lower than in the other groups. The apparent phosphorus absorption of test diet groups was significantly higher (36.9 ± 11.12% for MAB20, 24.0 ± 6.20% for MAB40, 57.1 ± 5.22% for MAB60, and 57.4 ± 5.34% for MAB100) than that of the control (8.3 ± 0.15%). Concentrations of protein and ash in the whole body increased as MAB substitution in diets increased. The findings suggest that MAB could replace up to 28% of FM protein in diets for juvenile rainbow trout for 16 weeks without adverse growth effects.

Fish meal (FM) has been a major ingredient in fish diets because of its high protein quality and palatability. Substituting less expensive protein sources for high-price FM in salmonid feeds is one way to lower production costs (Hardy 1996). For this reason, many studies have been conducted to replace or reduce its inclusion in fish diets by various less expensive alternative animal and vegetable protein sources; however, each candidate has characteristics that make it inferior in some respect to high-quality FM (Hardy 1996; Mambrini et al. 1999).

Feather meal, meat and bone meal, poultry by-product meal, and blood meal, the supplementary protein sources commonly incorporated at low levels (5–15%) in practical fish feeds (Murai 1992), have individually been studied as an animal protein replacements for FM. These terrestrial animal by-product meals have a high protein level and favorable essential amino acids profiles, but they are deficient in one or more of the essential amino acids (NRC 1993). If the proper combinations of these by-products are used in fish diets, the quality of the diet is likely to be improved (Davies et al. 1989). Few studies that used combinations of feed ingredients as an FM replacement have been reported. Yamamoto et al. (1995) reported that combinations of meat meal and malt protein flour effectively improved the growth of and protein utilization by fingerling rainbow trout *Oncorhynchus mykiss* compared with the replacement of FM by each ingredient alone. El-Sayed (1998) reported that in Nile tilapia *Oreochromis niloticus* a blood meal and meat and bone meal mixture resulted in lower growth performances compared with a FM-based diet.

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TABLE 1.—Proximate composition (%) of fish meal, mixture of animal by-products (MAB), and feed ingredients used for MAB (dry-matter basis).

Ingredient	Protein	Lipid	Ash	Moisture
Fish meal (herring)	75.7	10.0	10.4	5.71
Fish meal (menhaden)	65.6	10.2	19.0	6.21
MAB	73.1	14.4	12.3	5.96
Leather meal	68.5	16.7	7.75	4.11
Meat and bone meal	55.5	18.0	23.0	4.39
Feather meal	88.7	8.20	3.45	9.79
Squid liver powder	51.2	20.6	6.60	10.1
Poultry by-product meal	68.0	16.7	15.1	3.63
Blood meal	90.5	0.74	2.30	8.23

Because of their high protein levels and economic impacts, leather meal and squid liver powder can also be good candidates for replacing FM in fish feeds. Leather meal is produced from the tanning process, which generates much greater quantities of by-products and wastes than leather product (Simeonova and Dalev 1996). One ton of wet salted hides yields only 200 kg of leather but over 600 kg of solid by-product, and the chemical composition of the by-products varies according to the processing method (Cabeza et al. 1998). Squid liver meal is one of the traditional protein sources in diets for penacid shrimp because of its high level of lipid and n-3 highly unsaturated fatty acid fraction (n denotes the position of the first double bond from the methyl end). Merican and Shim (1995) reported that squid liver meal is characterized by high digestibility of dry matter and total lipid in diets for adult *Penaeus monodon*.

Therefore, it is important to study the nutritional values of combinations of animal by-products in order to replace FM in commercial fish diets without compromising growth and feed efficiency. The purpose of this study was to evaluate juvenile rainbow trout fed diets containing graded substitutions of a mixture of animal by-products (MAB) for FM protein, specifically by examining their growth and feed utilization, the whole body composition of minerals, and apparent protein and phosphorus digestibility. As part of the digestibility estimation we tested whether leaching of nitrogen occurred from sedimentary feces by comparing digestibility estimates based on feces collected at different times.

Methods

Diets.—Five experimental diets were formulated to be isonitrogenous and isocaloric in terms of crude protein (45%) and gross energy (4,180 kcal/kg). The energy value of each diet was estimated on the basis of mammalian physiological fuel values, i.e., 4 kcal/g protein or carbohydrate and 9 kcal/g lipid (Lee and Putnam 1973; Garling and Wilson 1977). A mixture of animal by-products (MAB) used in this experiment was obtained from Pukyong National University, Pusan, Korea, and consisted of 25% meat and bone meal, 24.5% leather meal, 20% squid liver powder, 15% feather meal, 7.5% blood powder, 7.5% poultry by-product meal, and 0.25% each methionine and lysine (Table 1). The ratio of each ingredient for MAB was based on protein contents and amino acid compositions and cost analysis for economic benefits. For the experimental diets (Table 2), FM was replaced by MAB on the basis of crude protein as follows: control diet = 100% FM; MAB20 diet = 80% FM: 20% MAB; MAB40 diet = 60% FM: 40% MAB; MAB60 diet = 40% FM: 60% MAB; and MAB100 diet = 100% MAB. To balance for phosphorus, calcium phosphate (monobasic) was added. The calculated essential amino acid concentrations in the experimental diets met or exceeded those recommended by NRC (1993). The experimental diets were pelleted, freeze dried, and stored at −20°C until used. For digestibility tests, 0.5% chromic oxide was included in the diets.

Fish, facility, and feeding trial.—The feeding trial was performed at the Piketon Research and Extension Center aquaculture facility with juvenile rainbow trout averaging (±SD) 0.96 ± 0.07 g initial weight. Prior to the feeding trial, fish were fed a commercial diet for 2 weeks to allow for adjustment to the experimental conditions. Fish were randomly distributed into groups of 30 in each of 3 tanks per treatment. Each experimental diet was fed to triplicate groups of fish; feeding rates ranged from 4% of fish weight at the beginning to 2% at the end of the feeding trial (NRC 1993). The fish were fed three times per day, 7 d per week. The feeding trial was conducted for 16 weeks in 50-L flow-through circular fiberglass tanks supplied with well water at a flow rate of 1.8–2.0 L/min.

TABLE 2.—Composition (%) of control and experimental diets; MAB = mixture of animal by-products.

Ingredient or characteristic	Control	MAB20	MAB40	MAB60	MAB100
Fish meal, menhaden	20.00	16.00	12.00	8.00	0.00
Fish meal, herring	20.00	16.00	12.00	8.00	0.00
MAB[a]	0.00	7.73	15.46	23.19	38.65
Krill meal[b]	5.00	5.00	5.00	5.00	5.00
Wheat middlings	28.00	28.00	28.00	28.00	28.00
Corn gluten meal	11.60	11.60	11.60	11.60	11.60
Brewer's yeast	6.00	6.00	6.00	6.00	6.00
Calcium phosphate	0.00	0.50	1.00	1.50	2.50
Vitamin mixture[c]	0.50	0.50	0.50	0.50	0.50
Mineral mixture[d]	0.50	0.50	0.50	0.50	0.50
Vitamin C[e]	0.05	0.05	0.05	0.05	0.05
Choline	0.10	0.10	0.10	0.10	0.10
Fish oil, menhaden	8.00	7.80	7.60	7.40	7.06
Cellulose	0.25	0.22	0.19	0.16	0.04
Proximate composition					
Crude protein	45.3	45.1	44.8	44.9	43.8
Phosphorus	1.63	1.60	1.64	1.58	1.52
Lipid	16.9	16.7	16.9	16.8	17.5
Energy (Kcal/kg)[f]	4,180	4,178	4,177	4,176	4,180

[a] Composed of 25% meat and bone meal, 24.5% feather meal, 20% squid liver powder, 15% feather meal, 7.5% blood meal, 7.5% poultry by-product meal, and 0.25% each methionine and lysine.

[b] Krill hydrolysates; Specialty Marine Products, Ltd., West Vancouver, British Columbia, Canada.

[c] Roche Performance Premix composition (per gram of vitamin mixture): vitamin A = 2,646 IU; vitamin D_3 = 221 IU; vitamin E = 66.1 IU; vitamin B_{12} = 13 μg; riboflavin = 13.2 mg; niacin = 61.7 mg; D-pantothenic acid = 22.1 mg; menadione = 1.32 mg; folic acid = 1.76 mg; pyridoxine = 4.42 mg; thiamin = 7.95 mg; d-biotin = 0.31 mg (Hoffman-La Roche, Inc., Nutley, New Jersey).

[d] Five mg Se in the form of sodium selenite per kilogram Bernhart Tomarelli salt mixture (ICN Pharmaceuticals, Inc., Irvine, California).

[e] Phospitan C (Mg-L-ascorbyl-2-phosphate); Showa Denko K. K., Tokyo, Japan.

[f] Calculated based on the compositions of the ingredients used (NRC 1993).

Supplemental aeration was also provided to maintain dissolved oxygen at levels near saturation. Water temperature increased gradually from 8°C to 15°C during the experiment and a diurnal light: dark cycle was regulated at 12 h: 12 h. Total fish weight in each tank was monitored every 4 weeks to check growth and adjust feeding rate. Feeding was stopped 24 h prior to weighing.

Feces collection and protein and phosphorus absorption.—The indirect method was used to calculate the apparent digestibility coefficient (ADC) with 0.5% chromic oxide as an inert indicator (Cho and Kaushik 1990). The apparent digestibility coefficients for protein and phosphorus were calculated using the following formula:

$$ADC_{nutrient} = [1 - (NF/ND \times CrD/CrF)] \times 100,$$

where: NF = percent nutrient in feces, ND = percent nutrient in diet, CrD = percent chromic oxide in diet, and CrF = percent chromic oxide in feces. Feces were collected with a modified fecal collection system after 16 weeks of the feeding trial (Yamamoto et al. 1998). From week 12 of the feeding trial, fish were fed the 0.5% chromic oxide diets to facilitate the digestibility test. After 16 weeks of feeding trial, three groups of 20 fish per diet treatment were transferred to three 50-L collection tanks having a steep conical bottom connected to a feces collection chamber. Water flowed to the top and out at the bottom of the conical tanks at a rate of less than 0.4 L/min. Fish were prevented from stirring the feces by a circular net placed at the base of the tank. To test the apparent nutrient digestibilities of the five experimental diets, the fish were fed their last respective meal at 2000 hours, and the feces were collected the next day at 0800 hours. To compare apparent protein digestibility with different times of fecal collection (every 40 min, after 12 h, and 48 h), the feces of the control fish fed the FM-based diet were collected every 40 min for 7 h and again at 48 h after feeding. The collected feces were immediately frozen at −20°C and then stored at −80°C until analyzed.

Sample collection and analysis.—Analyses of crude protein, moisture, and ash in diets and whole

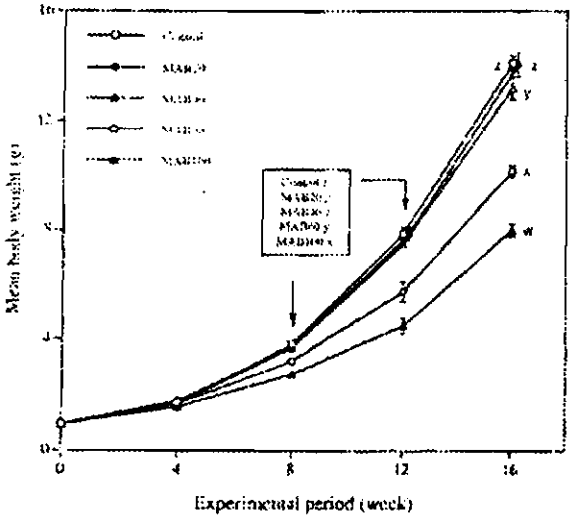

FIGURE 1.—Changes in mean body weight of juvenile rainbow trout fed five different diets (see Tables 1 and 2 for descriptions) for 16 weeks. Values are means ± SD of triplicate groups. Diets having different letters (z–w) are significantly different ($P < 0.05$); the boxed inset indicates statistical comparisons between groups at 8 and 12 weeks.

bodies were performed by standard procedures (AOAC 1995). Dietary lipid was determined according to Folch et al. (1957). At the end of the feeding trial, all fish were weighed and counted to calculate percent weight gain (body weight gain × 100/initial body weight), feed efficiency (body weight gain × 100/dry feed consumed), protein efficiency ratio (body weight gain/protein intake), specific growth rate ([$\log_e$ final body weight − $\log_e$ initial body weight] × 100/days), and survival. Diets and fish whole body were dry-ashed to determine mineral compositions by the inductively coupled plasma (ICP) emission spectrometry method (ARL-3560 Spectrometer; Applied Research Laboratories, Valencie, California) according to Watson and Isaac (1990). For chromium analysis, samples were digested with perchloric acid, then treated with the same method as other minerals.

Statistical analysis.—Data were subjected to one-way analysis of variance (ANOVA) using the SPSS statistical package (Version 9.0, SPSS Inc.). Percentage data were arcsine-transformed before analysis. A Fisher least significant difference (LSD) test was used to compare treatment means with $P = 0.05$. The possible replacement level of FM protein by MAB was determined by broken-line regression analysis (Robbins et al. 1979).

Results

Growth Performance and Whole Body Minerals

Average body weights of fish fed experimental diets during the 16-week period are shown in Figure 1. Fish growth rates began to differ in week 8 and became distinctly different between weeks 12 and 16. After the final week, fish fed diets containing 40, 60, and 100% MAB protein (diets MAB40, MAB60, and MAB100) exhibited significantly lower weight gain, feed efficiency, protein efficiency ratio, and specific growth rate compared with fish fed the control and MAB20 diets (Table 3). The regression equation for the broken-line analysis technique was $Y = 1558 - 8.527X$ ($r = 0.96$) and $Y = 1316$. The breakpoint at 28.3% MAB substitution level produced the least mean square error, and this value was determined as the acceptable substitution level of MAB. Interestingly, the whole body protein levels of fish increased as the MAB substitution in diets increased. No mortality was observed during the 16 weeks of the feeding trial. Lower diet acceptance was noticed in the fish fed MAB100 after the week 12.

TABLE 3.—Weight gain (WG), feed efficiency (FE), specific growth rate (SGR), protein efficiency ratio (PER), and whole body protein (WBP) of juvenile rainbow trout fed experimental and control diets for 16 weeks. Values are means ± SE of triplicate groups by diet; values in the same row followed by different letters are significantly different ($P < 0.05$). The acronym MAB = mixture of animal by-products.

Characteristic	Control	MAB20	MAB40	MAB60	MAB100
WG (%)[a]	1370 ± 17.0 z	1385 ± 26.9 z	1275 ± 23.5 y	959 ± 3.89 x	734 ± 5.59 w
FE (%)[b]	103 ± 1.09 z	104 ± 1.79 z	94.9 ± 1.70 y	80.3 ± 1.15 x	68.8 ± 0.67 w
SGR (%)[c]	2.40 ± 0.0 z	2.40 ± 0.02 z	2.34 ± 0.02 y	2.11 ± 0.00 x	1.89 ± 0.01 w
PER[d]	2.28 ± 0.02 z	2.31 ± 0.04 z	2.09 ± 0.04 y	1.78 ± 0.03 x	1.57 ± 0.01 w
WBP (%)[e]	56.1 ± 0.78 x	56.0 ± 0.70 x	56.5 ± 0.43 x	58.5 ± 0.58 y	60.4 ± 0.26 z

[a] Weight gain (%) = [(final weight − initial weight) × 100]/initial weight.
[b] Feed efficiency (%) = [body weight gain (g) × 100]/dry feed intake (g).
[c] Specific growth rate (%) = [($\log_e$ final weight − $\log_e$ initial weight) × 100]/d.
[d] Protein efficiency ratio = body weight gain (g)/protein intake (g).
[e] Whole body protein (%) = (protein concentration × 100)/dry whole body weight (g).

TABLE 4.—Total ash and mineral composition of the whole body of fish fed experimental and control diets for 16 weeks; MAB = mixture of animal by-products. Values are means ± SE of triplicate groups by diet; values in the same row without a letter in common are significantly different ($P < 0.05$).

Mineral	Initial	Control	MAB20	MAB40	MAB60	MAB100
Ash (%)		9.87 ± 0.23 yx	9.43 ± 0.50 x	9.93 ± 0.24 yx	10.4 ± 0.30 zy	11.3 ± 0.15 z
P (%)	1.47	1.59 ± 0.04 x	1.53 ± 0.03 x	1.64 ± 0.02 yx	1.72 ± 0.04 y	1.89 ± 0.08 z
K (%)	1.37	1.20 ± 0.01 y	1.21 ± 0.02 y	1.23 ± 0.05 y	1.28 ± 0.03 zy	1.36 ± 0.08 z
Ca (%)	1.52	1.76 ± 0.06 yx	1.61 ± 0.07 x	1.77 ± 0.05 yx	1.88 ± 0.06 zy	2.11 ± 0.12 z
Na (%)	0.38	0.30 ± 0.01 x	0.31 ± 0.01 x	0.33 ± 0.02 yx	0.35 ± 0.003 y	0.40 ± 0.02 z
Mg (%)	0.09	0.107 ± 0.00 y	0.107 ± 0.00 y	0.113 ± 0.00 zy	0.113 ± 0.00 zy	0.120 ± 0.00 z
Fe (μg/g)	227	117 ± 20.6 z	63.6 ± 5.36 y	99.6 ± 20.33 zy	73.3 ± 9.68 zy	67.0 ± 1.31 y
Mn (μg/g)	61.0	21.6 ± 6.48 z	7.02 ± 0.31 yx	13.5 ± 2.66 zy	11.2 ± 1.04 y	10.7 ± 1.23 y
Cu (μg/g)	1.23	1.72 ± 0.20 x	1.84 ± 0.16 x	2.61 ± 0.32 yx	3.44 ± 0.26 zy	3.87 ± 0.53 z
Zn (μg/g)	91.5	61.4 ± 0.81 yx	58.9 ± 2.35 x	65.2 ± 2.25 yx	68.3 ± 0.26 y	76.5 ± 3.70 z

Whole body ash levels increased as MAB substitution in diets increased and were significantly higher in MAB100 groups compared with the control (Table 4). All the individual mineral levels, except for Fe and Mn, followed the same trend as whole body ash. However, the levels of ash and individual minerals in feces collected 12 h after last feeding decreased as dietary ash and individual mineral levels decreased (Table 5), as related to increased MAB substitution in diets (Table 6).

Apparent Protein and Phosphorus Availability

After 16 weeks of feeding, apparent protein and phosphorus availabilities were determined in the feces collected 12 h after a meal (Figure 2). The apparent protein digestibility of diets containing MAB20 (85.8 ± 1.05%), MAB40 (83.1 ± 0.45%), and MAB60 (82.7 ± 2.67%) were not significantly different from that of the control diet (85.0 ± 1.88%), even though they produced lower growth rates than the control. However, apparent protein digestibility of the MAB100 diet (77.7 ± 4.41%) was significantly lower ($P < 0.05$) than that of the control diet. Contrary to the observed trend of apparent protein digestibility, test diets containing MAB exhibited significantly higher apparent phosphorus digestibility than the control diet. Apparent protein digestibility determined at fecal collection times of 12 h (85.0 ± 1.88%) and 48 h (87.4 ± 1.16%) did not differ significantly from that of feces collected every 40 min (86.0 ± 2.52%).

Discussion

Growth Performance and Whole Body Minerals

Based on the broken-line analysis, we found that approximately 30% of FM protein could be replaced by MAB, which would lead to growth rates in juvenile rainbow trout comparable to FM-based diets. This is significant because it is the first time, to our knowledge, that two new animal protein sources, leather meal and squid liver powder, have been shown to be effective MAB components in replacing FM in fish diets. Fish fed the FM-based control diet (1 herring: 1 menhaden) grew very well, showing comparable or higher specific growth rates (SGR, percent growth rate 47 d, 2.40 ± 0.01) than those reported in the literature. For instance, in an other rainbow trout study, Skonberg et al. (1997) found that similarly sized fish (initial

TABLE 5.—Ash and mineral composition of feces of the fish fed the experimental and control diets for 16 weeks; MAB = mixture of animal by-products. Values are means ± SE of triplicate groups; values in the same row without a letter in common are significantly different ($P < 0.05$).

Mineral	Control	MAB20	MAB40	MAB60	MAB100
Ash (%)	27.7 ± 0.33 z	22.7 ± 0.96 y	23.0 ± 1.16 y	17.0 ± 0.00 x	13.3 ± 0.33 w
P (%)	3.95 ± 0.08 z	3.39 ± 0.19 y	3.41 ± 0.18 y	2.11 ± 0.02 x	1.50 ± 0.04 w
K (%)	0.04 ± 0.007 zy	0.05 ± 0.009 z	0.04 ± 0.001 zy	0.03 ± 0.001 y	0.03 ± 0.007 zy
Ca (%)	6.45 ± 0.12 z	6.08 ± 0.34 z	5.96 ± 0.37 z	4.53 ± 0.12 y	3.43 ± 0.20 x
Na (%)	0.30 ± 0.02 yx	0.39 ± 0.01 zy	0.42 ± 0.03 z	0.33 ± 0.02 yx	0.26 ± 0.04 x
Mg (%)	0.74 ± 0.03 z	0.51 ± 0.03 x	0.66 ± 0.02 y	0.25 ± 0.02 w	0.20 ± 0.003 w
Fe (μg/g)	2,996 ± 473 z	815 ± 10.2 x	1,926 ± 187 y	1,029 ± 92.5 x	1,215 ± 85.6 yx
Mn (μg/g)	3,694 ± 614 z	435 ± 24.1 y	861 ± 16.6 y	429 ± 34.3 y	295 ± 22.0 y
Cu (μg/g)	24.0 ± 0.32 zy	17.4 ± 0.80 x	24.9 ± 1.76 z	23.5 ± 0.63 zy	21.9 ± 0.43 y
Zn (μg/g)	233 ± 4.73 z	225 ± 11.5 zy	217 ± 13.8 zy	201 ± 5.30 y	155 ± 6.70 x

TABLE 6.—Mineral composition of the experimental and control diets; MAB = mixture of animal by-products.

Mineral component	Control	MAB20	MAB30	MAB60	MAB100
P (%)	1.63	1.59	1.64	1.58	1.52
K (%)	0.96	0.92	0.92	0.88	0.81
Ca (%)	1.67	1.58	1.56	1.40	1.19
Na (%)	0.43	0.41	0.41	0.38	0.34
Mg (%)	0.32	0.30	0.30	0.29	0.26
Fe (μg/g)	235	259	297	336	404
Mn (μg/g)	103	101	105	103	102
Cu (μg/g)	16.5	14.9	14.7	17.3	17.7
Zn (μg/g)	89.3	84.3	86.6	84.9	81.4

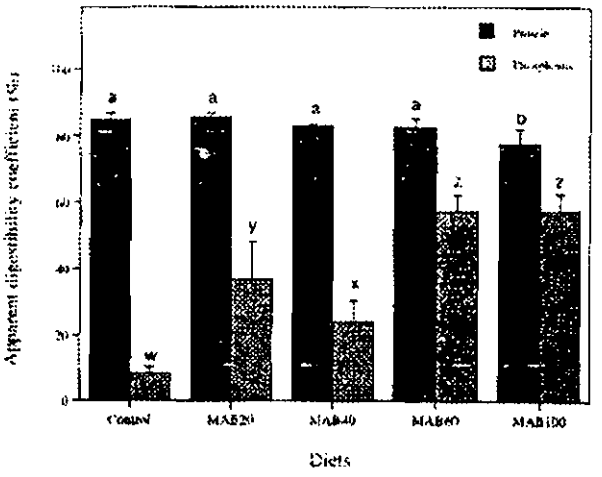

FIGURE 2.—Apparent digestibility coefficients for protein and phosphorus in juvenile rainbow trout fed experimental diets for 16 weeks. Feces were collected 12 h after the last feeding in the digestibility test. Values are means ± SD of triplicate groups. Bars having different letters (a–b for protein, z–w for phosphorus) are significantly different ($P < 0.05$).

weight, 1.8 g) fed a control diet (herring FM-based) for 8 weeks had an SGR of 1.42% SGR for restricted feeding (3–4%/ d) and 2.56% for ad libitum feeding at temperatures of 15–21°C. Rainbow trout, weighing 3 g, fed a menhaden FM-based diet ad libitum had an SGR of 2.77% after 13 weeks of feeding (Watanabe et al. 1993). Furthermore, the feeding period in our study was relatively long, compared with many other FM replacement studies of juvenile rainbow trout, because of rapid growth rates in the juveniles (Watanabe et al. 1993; Yamamoto et al. 1995; Stickney et al. 1996).

Most individual animal protein sources, such as meat and bone meal, feather meal, blood meal, and poultry by-product meal, have been able to replace less than 50% of FM in diets of salmonids. Fowler (1991) found that poultry by-product meal could compose 20% of a practical diet for chinook salmon *Oncorhynchus tshawytscha*, with concurrent reduction of the FM by 50%, without impairing growth and feed efficiency. Feather meal was reported as a minor replacer for FM in diets for coho salmon *O. kisutch* (Higgs et al. 1979), chinook salmon (Fowler 1982), and Nile tilapia (Bishop et al. 1995). High levels of meat and bone meal in fish diets showed different results in different fish species. Blood meal was also studied in rainbow trout (Luzier et al. 1995), eel (Lee and Bai 1997), and tilapia (Lee and Bai 1998). When FM is replaced with combined ingredients, the interpretation of results is difficult because many interactions between nutrients may be involved in nutrient metabolism. In our study, approximately 30% of MAB protein was able to replace FM protein in juvenile rainbow trout diets, showing that in the group with 40% MAB the growth rate decrease was less than 7% of the control. The lower growth performance is probably related to the inferior quality of the animal protein sources, but feather meal and squid liver powder seem to have potential as animal protein sources in rainbow trout feed.

Sugiura et al. (1998) reported that the levels of Ca and ash in salmonid diets formulated with animal by-product sources were inversely correlated to the percentage of net absorption of Ca, Fe, and Mn, indicating possible antagonistic interactions of Ca with these minerals. Our results were similar to that of Sugiura et al. (1998). In our study, ash and some mineral whole body concentrations (Table 4) were inversely correlated with those of the experimental diets (Table 6). The whole body concentrations of Ca, K, Na, and Mg were inversely correlated with dietary levels of Ca ($r = -0.89$), K ($r = -0.98$), Na ($r = -0.98$), and Mg ($r = -0.91$; $P < 0.05$ for all). However, the levels of ash and Ca in feces showed a positive correlation with the dietary ash ($r = 0.94$; $P < 0.05$) and Ca ($r = 0.99$; $P < 0.01$). A similar trend was observed in other mineral levels between diet and feces, even though the positive correlations were not significant ($P > 0.05$). This result confirmed data of Sugiura et al. (1998), who reported that fecal nutrient losses were positively correlated ($P < 0.05$) to nutrient intake, except for protein, Na, K, and Zn.

Apparent Protein and Phosphorus Availability

Apparent protein digestibility was not significantly different for up to 60% replacement of FM protein compared with that of the control diet, even

though weight gain was lower in the 40% and 60% replacement groups than in the control. This result may indicate that apparent protein digestibility of MAB is slightly lower than that of fish meal in juvenile trout. Although FM and MAB were not the only protein sources in the diets, the other protein sources (e.g., wheat middlings, corn gluten meal, yeast, and krill meal) were incorporated in all the diets at the same levels. Watanabe and Pongmaneerat (1991) reported that rainbow trout had lower protein digestibility of meat and bone meal (78%) than of white (92%) and brown (90%) FM. Robaina et al. (1997) mentioned that poor digestibility of meat and bone meal in diets of gilthead seabream *Sparus aurata* was due to its high ash content. The reason for the lower apparent protein digestibility of MAB in our study might be attributed to the poor amino acid profile rather than the high ash content of meat and bone meal (the major ingredient in MAB) because the ash content of MAB was lower than that of FM (Table 1).

Higher apparent phosphorus availabilities were found in the test diet groups than the control group (Figure 2). The reason for this seems to be due to supplementation of highly absorbable monobasic calcium phosphate in the test diets (NRC 1993). The availability of phosphorus from FM, which is primarily of bone origin, is generally lower than that of certain other high-protein feed ingredients, such as casein and yeast. Therefore, our findings suggest that using a mixture of animal by-products might improve phosphorus availability in juvenile trout diets. Furthermore, an MAB could have an advantage in reducing phosphorus release into the water, as indicated in feces phosphorus concentrations (Table 5). This is an important consideration because of the effect of aquacultural phosphorus discharge on stream eutrophication (Hardy 1999).

Feces collection frequently results in nutrients leaching into the water and leads to overestimation of digestibility: approximately 5–8% higher values than those determined by using fecal stripping or dissection methods (Brown 1993; Hajen et al. 1993; Watanabe et al. 1996; Allan et al. 1999). However, Allan et al. (1999) reported that stripping was not a suitable method for collecting digesta from juvenile silver perch *Bidyanus bidyanus* smaller than 10 g, and dissection methods exhibited considerably lower values than collection methods with rapid settlement. In our study, we used a collection method employing rapid settlement (Yamamoto et al. 1998) and determined apparent protein digestibility from feces collected at three different schedules (every 40 min, 12 h, and 48 h). Feces collected every 40 min retained their original bound form. However, we found some loosened of fecal form at 12 h and more at 72 h collections, but the related average values of protein digestibility were not statistically significant. This is supported by Allan et al. (1999), who reported similar digestibility from feces collected at different times (2, 6, 12, and 18 h), and by Satoh et al. (1992), who reported minimal differences in protein and lipid digestibility derived from feces collected at 3, 6, 9, 12, and 15 h after rapid settlement in a fecal collection chamber. Therefore, our findings suggests that, with rapid feces settlement systems, fecal collection times of 40 min to 48 h do not result in different protein digestibility values.

Based on the wholesale market price of 1998, fish meal (menhaden:herring, 1:1) used in the present study was US$ 2.51/kg and the estimated cost for MAB was 0.56/kg (Jack Bardall, OARDC, Wooster, Ohio, personal communication). Therefore, we expect that at least $ 0.22/kg of juvenile rainbow trout feed could be saved if 30% of FM protein is replaced by MAB. Use of MAB in fish diets as a FM replacement could be more cost-effective in the future if the price of FM continues to rise.

In conclusion, our findings suggest that MAB could replace approximately 30% of FM protein in diets for juvenile rainbow trout without adverse growth effects for up to 16 weeks. Also, fecal collections within 48 h of last feedings should not significantly overestimate apparent protein digestibility values. Further research will be required to determine the feasibility of using MAB composed of different combinations of ingredients and examine affects on larger sizes of fish.

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Short Course
in
Physiology of Fish Nutrition

July 17 to 21, 2006.

Escuela de Ciencias del Mar, Pontificia Universidad Católica de Valparaíso,
Av. Altamirano 1480, Valparaíso.

Professor: Dr. Charles Bai, Ph.D., Nutrition/Physiological Chemistry. (See attached CV)

Scope of the course.

This course deals with fish oriented Fish Nutritional Physiology. Nutritional and physiological implications will be looked at, to integrate structure and functions of nutrients in Fish Physio logy.

Units and Topics of the course.

Unit I.-
Introduction: 1. Define Nutritional Sciences, Biochemistry and Animal Physiology
2. Classification of Nutritionists and Nutrients
1-1. Definition of Fish Nutrition and Biochemistry:
1-2. Definition of Physiology:
1-3. Important subjects of physiology:

2-1. Nutritionists:
2-2. Nutrients:
2-2-1. Major nutrients groups: Protein, Lipids, Carbohydrates, Vitamins, Minerals and Water

Unit II.-
Energy metabolism: 1. Define the basic units
2. Energy partitioning and nomenclatures
3. Advantages of E metabolism in Fish
1-1. Calorie
1-2. Joule
2-1. Energy partitioning
2-2. Nomenclatures
3. Advantages of Energy metabolism in Fish

Unit III.-
Nutrients metabolism: 1. Protein & amino acids
2. Ammonia toxicity
1-1. Protein's structures, function and roles
1-2. Evaluation of protein quality
1-3. Nitrogen excretion (urea, uric acids, ammonia)

1-4. Amino acids' structure function
1-5. Definition of Essential Amino Acids (EAA) and NEAA
1-6. Definition of Biochemical Amino Acids and the Definition of Life Status
2-1. Ammonia Toxicity via TCA
2-2. via Neuro-transmitter
2-3. via unbalance of Cation in and out side of Cell (Na-K ATPase)

Unit IV.-

Nutrients metabolism: 1. Lipids and fatty acids
2. Carbohydrates
3. Vitamins
4. Minerals

1-1. Utilization, Classification and Function of Lipids
1-2. Classification, Properties and Structures of Fatty acids
1-3. Definition and Synthesis pathway of Essential Fatty Acids
2-1. Classification and Function of Carbohydrates
2-2. Properties and Structures of Carbohydrates
2-3. Essentiality of Carbohydrates and New Concept of Carbohydrates in Nutrition
3-1. Classification and Function of Vitamins
3-2. Properties and Structures of Fat Soluble Vitamins
3-3. Properties and Structures of Water Soluble Vitamins
4-1. Definition and Function of Minerals
4-2. Major Minerals
4-3. Semi-essential Minerals

Unit V.-

Feeds, Feed stuffs and Feed formulation: 1. Feeds and Feed stuffs
2. Feed formulations and practice

1-1. Classification of Feeds (FAO)
1-2. International Feed Number(My Book and FAO)
1-3. Quality Control requirements for Fish meal and Fish oil (My Book and FAO)
2-1. Basic concepts of computer feed formulation
2-2. Feed formulation practice I with 2-4 feeds stuffs for flounder(tables)
2-3. Feed formulation practice II
2-4. Feed Formulation practice III

Uint VI.-

Aquaculture and New area of research: 1. Direction of future aquaculture
2. Fish nutrition research review
(1) Develop new species diets
(2) Develop low pollution diet
(3) New techniques for research

1-1 Current status and future prospects of World Aquaculture
2-1 Develop new species diets (Korean rockfish)
2-2 Develop low pollution diets (Flounder)
2-3-1 New techniques for research (Operation Techniques in trout)
2-3-2

Unit I Introduction:

1. Define Nutritional Sciences, Biochemistry and Animal Physiology
2. Classification of Nutritionists and Nutrients

1-1. Definition of Fish Nutrition and Biochemistry:
1-2. Definition of Physiology:
1-3. Important subjects of physiology:
2-1. Nutritionists:
2-2. Nutrients:
2-2-1. Major nutrients groups: Protein, Lipids, Carbohydrates, Vitamins and Minerals

1-1. Definition of Fish Nutrition and Biochemistry

Nutrition is the study of nutrients metabolism in living organisms. This covers the ingestion of food, digestion, absorption and utilization of the ingested food and excretion of waste materials.

Fish Nutrition: Fish Nutrition is the study of nutrient metabolism in fish, which includes the ingestion of food by the fish, digestion, absorption and utilization of the ingested food and excretion of waste materials.

Two different approaches to study fish nutrition

(1) As a fish biologist, physiologist, or ecologist studying the nutritional aspects of fishes. This approach was used very often in field studies, i.e. food chain, stomach content analysis, prey selection, optimum foraging, feeding behavior, etc. to monitor and describe the feeding and nutrition of fishes.
(2) As a nutritionist just happen to use fish as an experimental model. This approach tends to rely heavily on growth trials to determine the essential nutrient requirements, utilization of nutrients, and diet formulations, etc. for fishes. They usually conducted under well controlled conditions.

Biochemistry: Biochemistry is the study of chemical reactions occurring in living organisms.

1-2. Definition of Physiology

The study of how living organisms function including such processes as nutrition, movement and reproduction. The branch of biology dealing with the functions and activities of living organisms and their parts, including all physical and chemical processes is known as physiology.

1-3. Important subjects of physiology:

1-3-1. Digestive physiology of animal:

Although there are numerous anatomical differences among different species, the principles of digestion and absorption are quite similar on a physiological basis. The process of digestion is a coordinated combination of physical, chemical and enzymatic activities. Digestion starts from the mouth (as soon as food is taken into the mouth) and ends at anus (when feces are excreted from the anus). In stomach, hydrolysis and break down of compounds occur at low pH (in presence of HCl and other acids). Here proteolytic enzyme (mostly pepsin) splits protein molecule. In non-ruminent animals, the small intestine is the primary site of both digestion and absorption. Here in an alkaline environment, proteolytic enzymes (from pancreas and intestine), amylases (from pancreas and intestine) and lipases (from pancreas and liver) split protein, carbohydrate and lipid, respectively. Then absorption can be taken place by the following three ways:

- Diffusion into mucosal cells
- Phagocytosis/pinocytosis by mucosal cells
- Active transport via carrier molecules

After absorption, the undigested fecal materials are excreted through anus.

1-3-2. Circulatory system

The circulatory system (sometimes cardiovascular system) is made up of the vessels and the muscles that help and control the flow of the blood around the body. This process is called circulation. The main parts of the system are the heart, arteries, capillaries and veins. As blood begins to circulate, it leaves the heart from the left ventricle and goes into the aorta. The aorta is the largest artery in the body. The blood leaving the aorta is full of oxygen. This is important for the cells in the brain and the body to do their work. The oxygen rich blood travels throughout the body in its system of arteries into the smallest arterioles. On its way back to the heart, the blood travels through a system of veins. As it reaches the lungs, the carbon dioxide (a waste product) is removed from the blood and replaced with fresh oxygen that we have inhaled through the lungs.

This is the general feature of blood circulation in almost all animals although there are some differences from species to species. There are three types of circulatory systems:

I) No circulatory system:
Flatworm, for example, has no blood circulation system. Their cell uptakes nutrients, water and oxygen without the need of a transport system.

II) Open circulatory system:
Some invertebrates (like mollusks and arthropods) belong to this type of system where there are some cavities, hemocoel, filled with hemolymph in their body. Hemolymph is not only blood rather it is a mixture of blood and intestinal fluid. This cavity bathes the organ directly and the organ gets oxygen and nutrients thereby.

III) Closed circulatory system:
In this system blood never leaves the system of blood vessels consisting of arteries, capillaries and veins; hence this name. All vertebrates belong to this system.

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1-3-3. Respirations
1-3-4. Neurophysiology
1-3-5. Reproductive physiology
1-3-6. Systemic physiology
a. Form and movement b. Thermal regulation c. Osmoregulation d. Ionic regulation e. Freezing resistance f. Acid base balance

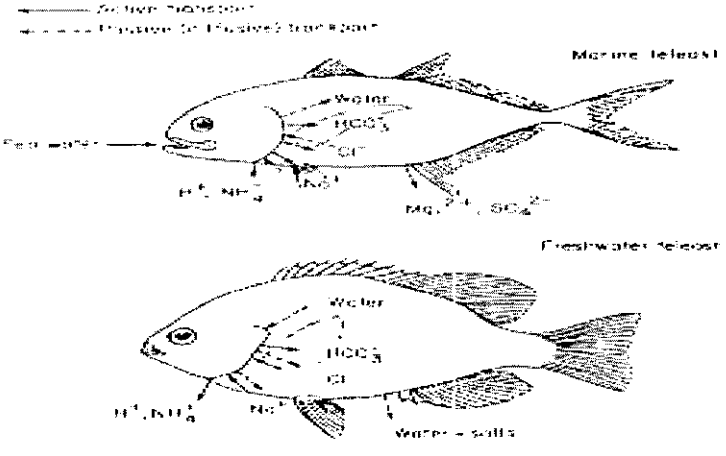


Figure 6.1 Passive and active routes of salt and water exchange.

1-3-7. Pathophysiology

2-1. Nutritionists

The scientists who are working with almost all aspects of nutritional sciences are called nutritionists.

1. Nutritionist
A. Human nutritionist
B. Animal nutritionist
- (1) Ruminent aminal nutritionists
- (2) Monogastric animal nutritionist
    - a. Poultry/bird nutritionists
    - b. Companion animal nutrionist
    - c. Fish nutrionist (finfish, shellfish, mollusc etc)
    - d. Laboratory animal nutritionist
    - e. Other specific type of animal nutritionist

2-2. Nutrients

2-2-1. Major nutrients groups

The nutrients in fish nutrition can be classified into six nutrient groups as follows:

Proteins: The need for proteins by fish is actually the need for the essential amino acids (Arginine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Threonine, Tryptophan and Valine) and the non-essential amino acids (Alanine, Asparagine, Aspartate, Cysteine, Glutamate, Glutamine, Glycine, Proline, Serine and Tyrosine). Proteins serve as one of the three energy sources among other roles and are required in large quantities.

Lipids: lipids are a chemically diverse group of compounds, the common and defining feature of which is their insolubility in water. The biological functions of the lipids in fish are diverse. The fats and oils used as stored forms of energy in fish are highly reduced compounds, derivatives of fatty acids. The essential fatty acids (EFA) for fish include linoleic acid (18:2n-6) and/or alpha linolenic acid (18:3n-3) for freshwater fish as well as Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA) for marine species.

Carbohydrates: The availability of carbohydrates as energy source for fish varies from species to species but is generally low. Carbohydrates include monosaccharide, disaccharides, oligosaccharides and polysaccharides.

Vitamins: Vitamins are organic molecules that act as cofactors or substrates in some metabolic reactions. They are generally required in relatively small amounts in the diet and, if present in inadequate amounts, may result in nutrition-related diseases, poor growth or increased susceptibility to infections. They include the water-soluble vitamins

(Thiamin, Riboflavin, Pyridoxine, Pantothenic acid, Niacin, Biotin, Folic acid, Cyanocobalamin, Ascorbic acid, Inositol and Choline) as well as the fat-soluble vitamins (Vitamins A, D, E and K).

Minerals: These are involved in formation of skeletal structure, maintenance of colloidal systems (osmotic pressure, viscosity, diffusion), and regulation of acid-base equilibrium. They are important components of hormones, enzymes, and activators of enzymes. They include the macroelements (Potassium, Sodium, Calcium, Magnesium, Phosphorus, Chloide and Sulfur) and the microelements (Iron, Copper, Zinc, Manganese, Molybdenum and Selenium).

Water: Water is the most abundant substance in living systems. Water pervades all portion of every cell of fish and is the medium in which the transport of nutrients, the enzyme-catalyzed reactions of metabolism, and the transfer of chemical energy occur. Water is obtained from the natural habitat of fish.

Unit II
Energy metabolism:

1.Define the basic units
2. Energy partitioning and nomenclatures
3. Advantages of E metabolism in Fish

1-1. Calorie
1-2. Joule
2-1. Energy partitioning
2-2. Nomenclatures
3. Advantages of energy metabolism in fish

1-1. Calorie

One calorie is defined as the amount of heat energy required to increase the temperature of one gram of water 1°C from 14.5 to 15.5°C.

1 calorie (cal) = 4.18 joules (J)

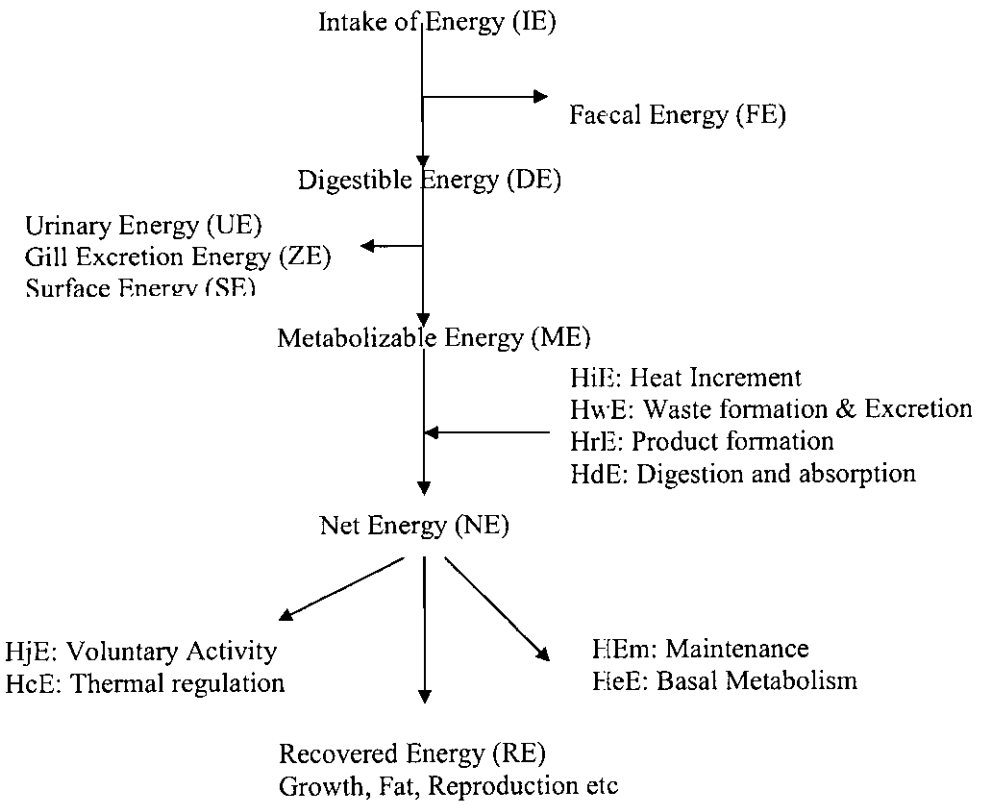
1-2. Joule

The standard international unit for expressing mechanical, chemical or electrical energy, as well as the concept of heat. One joule is defined as the amount of energy exerted when a force of one newton (standard international unit of force, force required to accelerate 1m per second with 1 kg of material) is applied over a displacement of one meter. One joule is the equivalent of one watt of power radiated or dissipated for one second.

4.18 joules = 1 calorie

2-1. Energy partitioning

Energy is required for maintaining the physiochemical environment of the intact animal and for sustaining the electromechanical activities that define the organism. The major body store of energy is adenosine triphosphate (ATP) and other high-energy phosphate bonds. Animals consume food for energy. This food goes through an intricate metabolic pathway and eventually produces energy as ATP. In mitochondria (power house of cell), ATP burns into energy as required by the different organelles /organs of the body and distributed accordingly. This energy distribution or partition can be, for easy understanding, shown in the below adapted from the previous publication (NRC, 1981).



2-2. Nomenclatures

Intake energy (IE) is the gross energy (GE) consumed by an animal in its food. The majority of intake energy is present in the form of carbohydrate, protein and/or lipid.

Fecal energy (FE) is the gross energy of the faeces. Faeces consist of undigested food and metabolic products, which may include sloughed gut epithelial cells, digestive enzymes and excretory products.

The digested energy within a food is called the apparent digestible energy (DE) and is determined as the energy in food minus the energy in faeces:

$$\mathbf{DE = IE - FE}$$

Urinary energy (UE) is the gross total energy in urinary products. It includes energy of compounds absorbed from the food but not utilized and the energy of products of metabolic processes such as ammonia.

Gill excretion energy (ZE) is the gross energy of the compound excreted through the gills of aquatic animals. Its equivalent in mammalian energetics is the energy of the compounds excreted through the lungs. However, in mammals this energy is extremely low and generally not considered. In fish ZE can be quite high and may constitute a major component of the energy balance of an aquatic organism.

Surface energy (SE) is the energy lost from the surface of an organism. In aquatic organisms, this may take the form of mucus or scales sloughed from the skin.

Metabolizable energy (ME) is the energy in the food less the energy lost in faeces, urine and through excretion from the gills. It is the energy available for the conduct of the metabolic processes of an animal. It may be described by the equation:

$$\mathbf{ME = DE - (UE + ZE + SE)}$$

$$\mathbf{ME = (IE - FE) - (UE + ZE + SE)}$$

The difference between ME and energy recovered as growth and/or reproductive products (RE) is energy lost as heat (HE). Heat loss occurs primarily by two processes: the heat increment of feeding (HiE) and maintenance heat loss (HEm).

The HiE is the increase in heat production subsequent to ingestion of feed. The factors contributing to HiE are the digestion and absorption processes (HdE), the transformation and interconversion of the substrates and their retention in tissues (HrE), and the formation and excretion of metabolic wastes (HwE).

Net energy is the energy that is useful to the animal for maintenance and growth. It is the Metabolizable energy (ME) less the heat increment of feeding (HiE).

Maintenance energy (Hem) is that required to maintain those functions of the body immediately essential to life. A major portion of this maintenance energy is spent for basal metabolism (HeE), such as respiration, transport of ions and metabolites, body constituent turnover, and circulation. A smaller portion is spent for voluntary or resting activity (HjE) and, in the case of homeothermic animals, thermoregulation of body temperature. Since fish do not regulate body temperature and they expend less energy in

maintaining position in the water than do terrestrial animals in maintaining their posture, the Hem requirement of fish is lower than for homeotherms.

Retained energy (RE) is that portion of the energy contained in the food that is retained as part of the body or voided as a useful product such as gametes. The aim of nutrition in aquaculture is to maximize RE and minimize all other energy losses in a cost-effective manner, whilst maintaining an acceptable body composition of the end product.

The energy balance of an organism therefore can be described by the equation:
IE (or GE) = FE + DE
IE = FE + (UE, ZE, SE) + ME
IE = FE + (UE, ZE, SE) + (HiE, HwE, HrE, HdE) + NE
IE = FE + (UE, ZE, SE) + (HiE, HwE, HrE, HdE) + (HEm, HcE, HjE, HcE) + RE

3-1. Advantages of energy metabolism in fish
Advantages of aquaculture from the nutritional point of view. It requires less energy to produce fishes because:

1) The specific gravity of fishes is close to 1 and they have a neutral buoyancy in water, thus require less energy to maintain their body weight. Due to the buoyancy of water, fish spend less energy in maintaining position in the water than do terrestrial animals in maintaining their posture.
2) Because most fish, as cold-blooded vertebrates or ectotherms, within the zone of thermal neutrality do not regulate their body temperature; hence, the heat of thermal regulation is insignificant. However, warm-blooded vertebrates have to keep their body temperature within a certain range (i.e. 36.5°C for human).
3) Fish are ammonotelic animals require less energy to excrete their nitrogen waste than uricotelic or ureotelic animals converting ammonia to uric acid or urea. Protein is not cleam fuel. Left over nitrogen should be excreted outside of the animal body. Most mammal produce urea through urea cycle, and birds and reptile produce uric acid (pentose pathway)
4) Fish have better feed efficiency (fish 1.2 to 2, Rainbow trout 1.5; Catfish 1.8; poultry 2.0; pig 4.0; Cow 8 to 20).
5) Fish have a high fecundity and reproductive performance such as more spawning frequency and more eggs than do terrestrial animals.

Unit III
Nutrients metabolism:

1. Protein & amino acids
2. Ammonia toxicity

1-1. Protein's structures, function and roles
1-2. Evaluation of protein quality
1-3. Nitrogen excretion (urea, uric acids, ammonia)
1-4. Amino acids' structure function
1-5. Definition of Essential Amino Acids (EAA) and NEAA
1-6. Definition of Biochemical Amino Acids and the Definition of Life Status
2-1. Ammonia Toxicity via TCA
2-2. via Neuro-transmitter
2-3. via unbalance of Cation in and out side of Cell (Na-K ATPase)

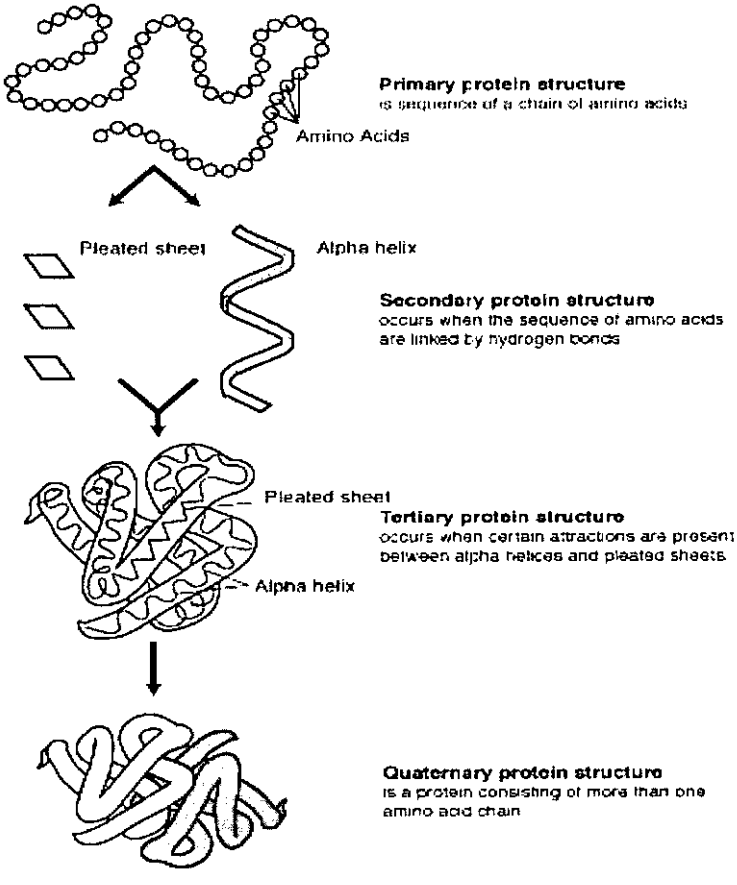
1-1. Protein's structures, function and roles

Protein's structures: Proteins are not linear molecules as suggested when we write out a "string" of amino acid sequence, -Lys-Ala-Pro-Met-Gly- etc., for example. Rather, this "string" folds into an intricate three-dimensional structure that is unique to each protein. It is this three-dimensional structure that allows proteins to function. Thus in order to understand the details of protein function, one must understand protein structure.

Protein structure is broken down into four levels:

- **Primary structure:** The **linear sequence** of amino acids in a protein chain determined by the genetic information of the gene for each protein. It results from the covalent bonding between the amino acids in the chain (peptide bonds). Each protein has a different primary structure with different amino acids in different places along the chain.
- **Secondary structure: Folding** of the primary structure of a protein into an α-helix or a β-pleated sheet. Folding is maintained by hydrogen bonds between the amide hydrogen and the carbonyl oxygen of the peptide bond. Different regions of a protein chain may have different types of secondary structure.
- **Tertiary structure:** The globular, **three-dimensional structure** of a protein that results from folding the regions of secondary structure. This folding occurs spontaneously as a result of interactions of the side chains or R groups of the amino acids. The forces that help maintain the tertiary structure of a protein are hydrogen bond, ionic bond, disulfide bond, and hydrophobic attraction.

- **Quaternary structure:** Involves the association of two or more polypeptide chains into a multi-subunit structure. Quaternary structure is the stable association of multiple polypeptide chains resulting in an active unit. Here also works the four forces to hold the quaternary structure stable eg. hydrogen bond (between polar amino acids), ionic bond (between oppositely charged amino acids), disulfide bond (also called disulfide bridge, a strong covalent bond between two sulfhydryl (-SH) groups), and hydrophobic attraction (between non-polar amino acids). For example- the protein hemoglobin has quaternary structure.



***Roles of dietary protein*:**

- supply essential amino acids
- supply nitrogen to synthesize non-essential amino acids
- supply sulfur

*** Only protein can supply nitrogen and sulfur. These two elements are unavailable from fats and carbohydrates, the other major classes of food molecules.

In addition to their dietary importance, the proteins carry out most of the work in a cell. The biological functions performed by protein are as follows:

Function	Description	Examples
Antibody	Antibodies bind to specific foreign particles, such as viruses and bacteria, to help protect the body.	Immunoglobulin G (IgG)
Enzyme	Enzymes carry out almost all of the thousands of chemical reactions that take place in cells. They also assist with the formation of new molecules by reading the genetic information stored in DNA.	Phenylalanine hydroxylase
Messenger	Messenger proteins, such as some types of hormones, transmit signals to coordinate biological processes between different cells, tissues, and organs.	Growth hormone
Structure	Provide mechanical support to large animals and provide them with their outer coverings.	Keratin
Transport/storage molecule	These proteins bind and carry atoms and small molecules within cells and throughout the body.	Ferritin

1-2. Evaluation of protein quality

There are different methods for protein quality evaluation which are listed below:

1. Protein efficiency ratio (PER) = $\frac{\text{gain in body weight (g)}}{\text{protein consumed (g)}}$

Values are normally in the 1-4 range. Value near 2 indicates poor quality protein whereas 4 indicates good quality protein.

2. Net protein utilization (NPU%) = $\frac{\text{protein (N) gain in fish (g)}}{\text{protein (N)intake in food (g)}} \times 100$

3. Relative nutritive value (RNV)

4. Biological value (BV) = $\frac{\text{N retained} \times 100}{\text{N absorbed}}$ (% absorbed N retained)

$$BV = \frac{\{\text{N intake} - (\text{fecal N} - \text{metabolic fecal N}) - (\text{urinary N} - \text{endogenous urinary N})\}}{\text{N intake} - (\text{fecal N} - \text{metabolic fecal N})}$$

Metabolic fecal N and endogenous urinary N are the quantity of N excreted while animal is fed a protein free diet.

$$\text{5. Chemical score (CS)} = \frac{\%\text{ limiting amino acid in expt. protein} \times 100}{\%\text{ same amino acid in reference protein}}$$

1-3. Nitrogen excretion (ammonia, urea and uric acids)

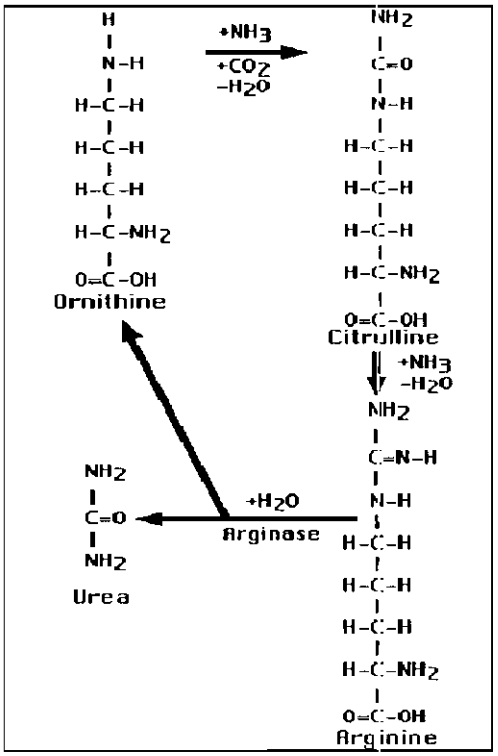
Ammonia, the product of oxidative deamination reactions, is toxic to the nervous system even small amounts and its accumulation rapidly causes death. Therefore it must be detoxified to a form which can be readily removed from the body. NH_3 represents 70 to 90% of the total nitrogenous waste in fish (Mommsen and Walsh, 1992) with 5 to 15% excreted as urea depending on the species of fish (Dosdat *et al.*, 1996). Maximum fish excrete NH_3 as nitrogenous waste rather than urea and uric acid. Synthesis of urea and uric acid from NH_3 consumes energy, thus mammals and birds derive less metabolic energy from amino acid catabolism than do fish. This is a beneficial aspect for fish and this may be a cause why fish use protein as well as amino acids for their major energy source. The marine bony fish, although living in water which is more concentrated than their body fluids, are able to osmoragulate by excreting salt through their gills. The elasmobranches (**Sharks**, **Skates**, and **Rays**) do not maintain the salinity of their body fluid in this way. Rather they produce urea from NH_3 and use high concentration of urea in their body fluids to prevent dehydration and salt accumulation. However, even fish do not have large concentrations of ammonia in the blood because it is excreted as the amide in glutamine. Glutamine is carried to a membrane in the gills near the surrounding water where a hydrolysis of the glutamine to glutamic acid releases the ammonia.

Birds, **insects**, **lizards** and **snakes** convert the waste products of protein metabolism as well as nucleic acid metabolism into **uric acid** (whitish portion of their excreta). Because of its low solubility in water, these animals are able to eliminate waste nitrogen with little loss of water. Urea is the major end product of nitrogen metabolism in humans and mammals. **Humans** excrete **urea** and secondly **uric acid** as nitrogenous waste. But, uric acid is the product of **nucleic acid**, not protein, metabolism. Uric acid is only slightly soluble in water and easily precipitates out of solution forming needlelike crystals of sodium urate. These contribute to the formation of kidney stones; produce the excruciating pain of **gout** when deposited in the joints. Most mammals have an enzyme — **uricase** — for breaking uric acid down into a soluble product.

The **urea cycle** or the ornithine cycle describes the conversion reactions of ammonia into urea. Since these reactions occur in the **liver**, the urea is then transported to the kidneys where it is excreted. The overall urea formation reaction is:

2 Ammonia + carbon dioxide + 3ATP ---> urea + water + 3 ADP

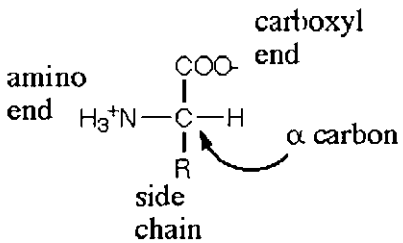
Urea



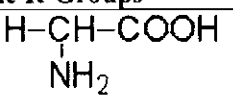
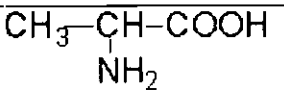
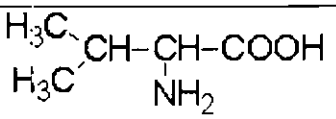
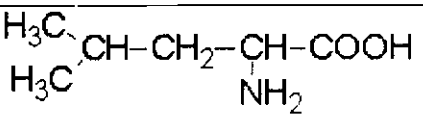
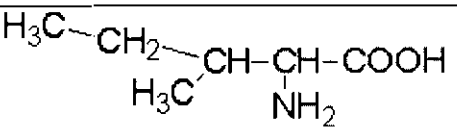
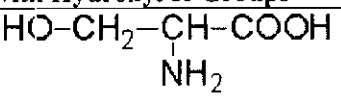
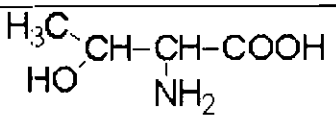
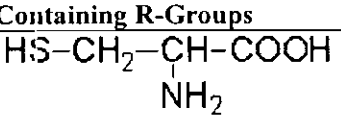
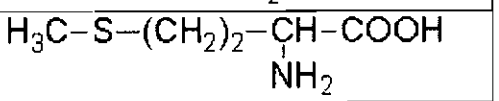
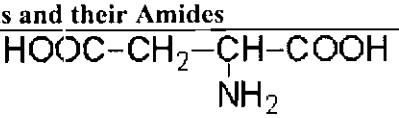
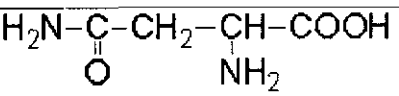
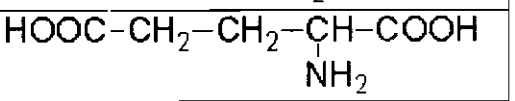
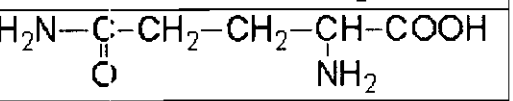
cycle

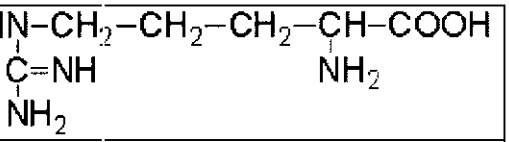
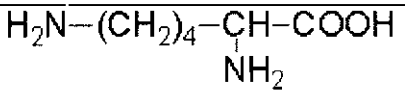
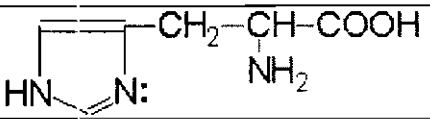
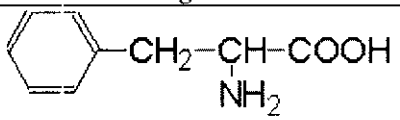
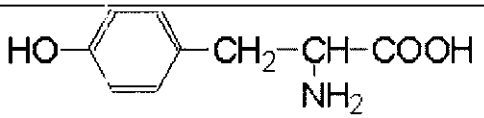
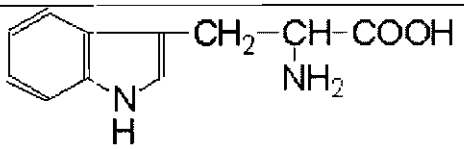
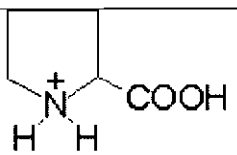
1-4. Amino acid's structure & function

Amino acid's structure: Amino acids are the building blocks of proteins. An α-amino acid consists of a central carbon atom, called the α carbon, linked to an amino group, a carboxylic aid group, a hydrogen atom, and a distinctive R group. All amino acids have the same general formula.



Amino Acid	Symbol	Structure*

Amino Acids with Aliphatic R-Groups		
Glycine	Gly - G	
Alanine	Ala - A	
Valine	Val - V	
Leucine	Leu - L	
Isoleucine	Ile - I	
Non-Aromatic Amino Acids with Hydroxyl R-Groups		
Serine	Ser - S	
Threonine	Thr - T	
Amino Acids with Sulfur-Containing R-Groups		
Cysteine	Cys - C	
Methionine	Met-M	
Acidic Amino Acids and their Amides		
Aspartic Acid	Asp - D	
Asparagine	Asn - N	
Glutamic Acid	Glu - E	
Glutamine	Gln - Q	
Basic Amino Acids		

Arginine	Arg - R	
Lysine	Lys - K	
Histidine	His - H	
Amino Acids with Aromatic Rings		
Phenylalanine	Phe - F	
Tyrosine	Tyr - Y	
Tryptophan	Trp-W	
Imino Acids		
Proline	Pro - P	

*Backbone of the amino acids is red, R-groups are black

Function of amino acids: Amino acids fulfill multiple metabolic functions in the human body.

- Amino acids act as building blocks for proteins and as precursors for hormones, neurotransmitters, antioxidants, nucleic acids and other complex body constituents.
- They also are used for energy once the body has metabolized all carbohydrate sources (glycogen).

Other than for protein synthesis, amino acids play the following roles:

Amino acids	Selective functions
Arginine	vasodilatation, radical scavenging
Arginine, BCAA, Glutamic Acid	NH3 detoxification and transport
Arginine, Glutamine	Growth hormone secretion
Cysteine, Arginine, Glutamine	Stimulation and stabilization of immune system
Cysteine, Glutamine	Body cell mass maintenance
Cysteine	Antioxidant; radical scavenging
Glutamine	Improved mental and physical performance
Methionine	Urine acidification, wound healing
Phenylalanine, Tyrosin	Precursor for hormones and neurotransmitters (dopamine)

***BCAA = branched chain amino acid

1-5. Definition of EAA and NEAA

Essential amino acid: Amino acids that can not be synthesized by humans and other vertebrates, and must be obtained from the diet. The essential amino acids (EAA) are synthesized in micro-organisms (bacteria and yeasts having N_2 fixation ability from atmosphere which lacks in higher organisms) and passed through the food chain until they reach us in our diet.

The essential amino acids are: Isoleucine, Leucine, Lysine, Methionine, Phenylaianine, Threonine, Tryptophan and Valine.

Nonessential amino acids: Amino acids that can be synthesized by humans and other vertebrates from simpler precursors, and are thus not required in the diet.

The non-essential amino acids are: Alanine, Arginine, Asparagine, Aspartic acid, Cysteine, Clutamic acid, Clutamine, Glycine, Histidine*, Proline, Serine and Tyrosine.

* Histidine is essential for babies but not for adults.

1-6. Definition of Biochemical amino acids and life status

Biochemical amino acids: It is believed to have 104 -- 110 amino acids available but among them only 20/22 plays important role in protein synthesis. These 20/22 amino acids are called Biochemical amino acids. Therefore, EAA + NEAA + Selenocysteine (21st) + Hydroxylysine (22nd) = Bicheminal amino acids.

Life status: The status of life is nothing but protein synthesis according to the genetic information.

2-1. Ammonia toxicity via TCA

Ammonia is extremely toxic to animal. Therefore, animal system is so designed that they readily convert ammonia into either urea or uric acid. Maximum fish excrete ammonia as it is rather than convert into urea or uric acid. If it is not, then what would be?
When NH_3 increases it reacts with α-ketoglutarate (α-KG), an intermediate product of TCA cycle in the presence of GDH (Glutamate dehydrogenase). GDH facilitates the following reaction and depletes α-KG from the running TCA cycle. Thus, TCA can not maintain the way of its normal running. It slows down and finally collapses.

α-ketoglutarate + NH_3 + NADH $\longleftrightarrow$ Glutamate + NAD

2-2. via neuro-transmitter

Glutamate increases at the same time NH_3 increases in the blood. Glutamate is the precursor for GABA (Gamma-aminobutyric acid), an inhibitory neurotransmitter found in the nervous systems of widely divergent species. Therefore, GABA increases substantially and affects the central nervous system (CNS). As a result, CNS loses all of its normal control.

2-3. via unbalance of cation in & out of the cell (NA-K ATPase)

For its normal function, cells need to have a balance of cation (Na^+ and K^+) in and out of it. K^+ is mainly needed inside the cells whereas outside Na^+. Na^+ K^+ATPase help maintain this balance. When NH_3 increases in the blood it can move freely in and out of the cells. In this case, Na^+ K^+ATPase get confusion and continue to pump. The balance state of cation of the cell becomes unbalance thereby.

Unit IV
Nutrients metabolism:

1. Lipids and fatty acids (2 hrs)
2. Carbohydrates (1.5 hrs)
3. Vitamins (2 hrs)

1-1. Utilization, Classification and Function of Lipids
1-2. Classification, Properties and Structures of Fatty acids
1-3. Definition and Synthesis pathway of Essential Fatty Acids
2-1. Classification and Function of Carbohydrates
2-2. Properties and Structures of Carbohydrates
2-3. Essentiality of Carbohydrates and New Concept of Carbohydrates in Nutrition
3-1. Definition, Classification, Function, Deficiency Symptoms and Sources of Vitamins
3-2. Properties and Structures of Fat Soluble Vitamins
3-3. Properties and Structures of Water Soluble Vitamins
4-1. Classification and Function of Minerals
4-2. Major Minerals
4-3. Semi-essential Minerals

1-1.Utilization, classification and function of lipids

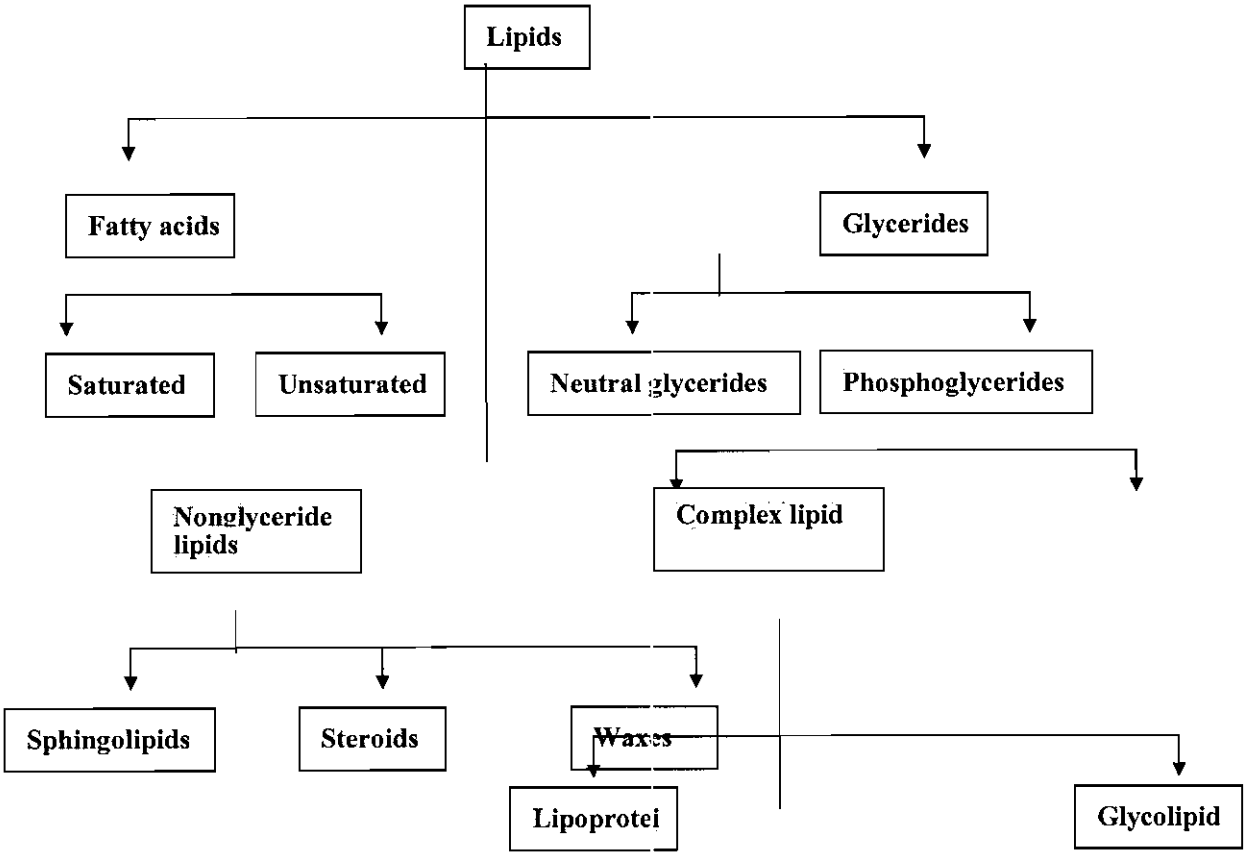
Digested by enzymes and emulsified (micelle formation), absorbed as fatty acids and resynthesized to triglycerides and transported in lymph as chylomicrons

Classification of lipid:

A. Simple lipids :
1. **Fats-esters of FA with glycerol oil-a fat in the liquid state.**
2. **Waxes-FA w/higer MW monohydri-alcohols**

B. Complex Lipeds
1. **Phospholipids (Sphingophospholipids)**
2. **Glycolipids (Glycosphingolipids), FA + Sphingosine + CHO**
3. **Other complex lipids (Lipo protein)**

C. Precursor and Derived lipids : FAs, Steroids, KBs



Functions of lipid:

Lipids serve many important functions in the animal body. Here is a brief summery of that.

- Lipids are an excellent source of energy for the body. When oxidized, 1g of fat gives 9 kilocalories of energy.
- Provide essential fatty acids
- Most of the energy stored in the body is in the form of lipids (triglycerides).
- Phospholipids are important constituent of cell membrane.

- ☙ The steroid hormones are critical chemical messengers that allow the tissues of the body to communicate with one another. The prostaglandins exert strong biological effects on both the cells that produce them and other cells of the body.
- ☙ Fat soluble vitamins (A, D, E & K) play very important roles in biological processes.
- ☙ Dietary fat serves as a carrier of the lipid-soluble vitamins.
- ☙ Lipids protect many vital organs from outer shocks.
- ☙ Subcutaneous fat serves to insulate the body from extremes of cold temperatures.

1-2.Classification, properties and structures of fatty acids

Fatty acid classification properties:

Based on number of double bond

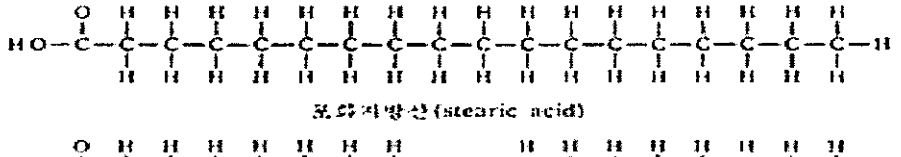
- ☙ Saturated fatty acid
  - No double bonds
  - Solid at room temperature
  - Implicated in coronary heart disease (CHD)

  Sources: Meats, dairy, tropical

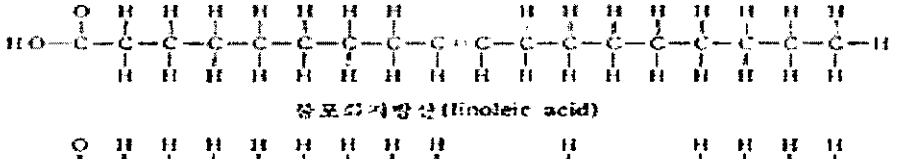
- ☙ Unsaturated fatty acid
  - Polyunsaturated fatty acid (PUFA): 2-5 double bonds
  - Highly unsaturated fatty acid (HUFA): >5 double bonds
    - Has double bonds
    - Liquid at room temperature
    - Less stable, prone to rancidity

  Sources: **Vegetables, legumes, fish**

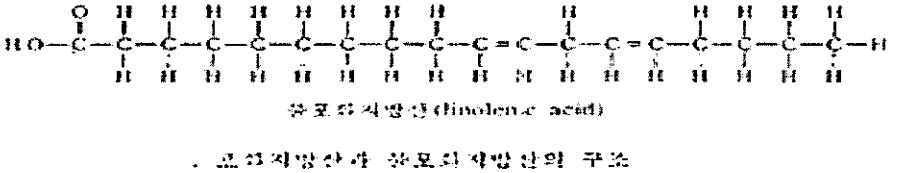
Structures of fatty acids:



포화지방산(stearic acid)



불포화지방산(linoleic acid)



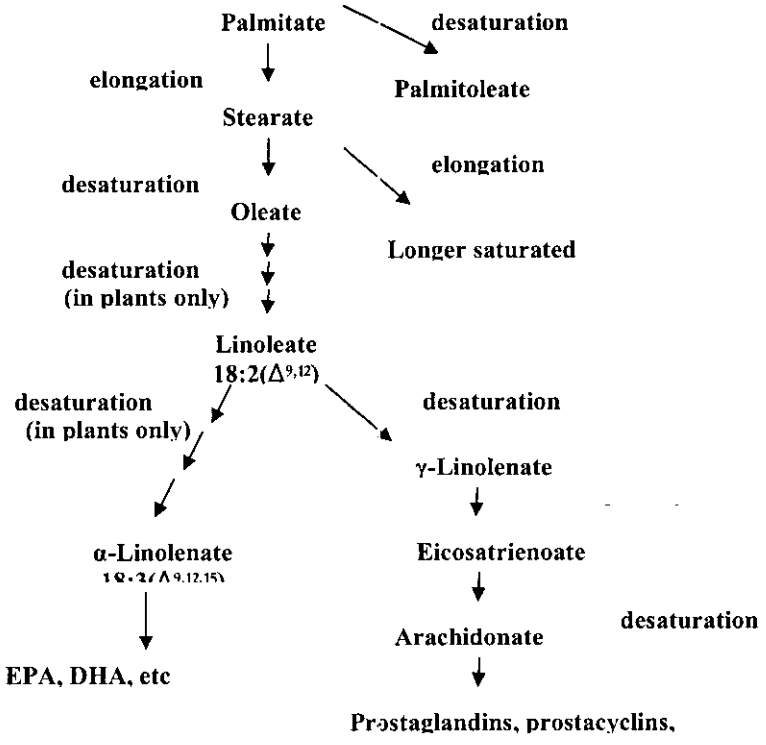
불포화지방산(linolenic acid)

. 포화지방산과 불포화지방산의 구조

1-3.Definition and synthesis pathway of essential fatty acid

Definition essential fatty acid:
A fatty acid that can not be synthesized in the body or that can not be made in sufficient quantities for the body's needs is called essential fatty acid (EFA). For instance, linolenic acid and linoleic acid are called EFA because they must be supplied in the diet.

Synthesis pathway of essential fatty acid:



2-1. Classification and function of carbohydrates

Classification of carbohydrates:

- Monosaccharides eg. Glucose, fructose, galactose
- Disaccharides eg. Sucrose, lactose, maltose
- Oligosaccharides eg. Maltodextrins
- Polysaccharides
  - I. Starch eg. Amylose
  - II. Non-starch eg. Cellulose, pectins

Function of carbohydrates:

Carbohydrates have 6 major functions within the body:

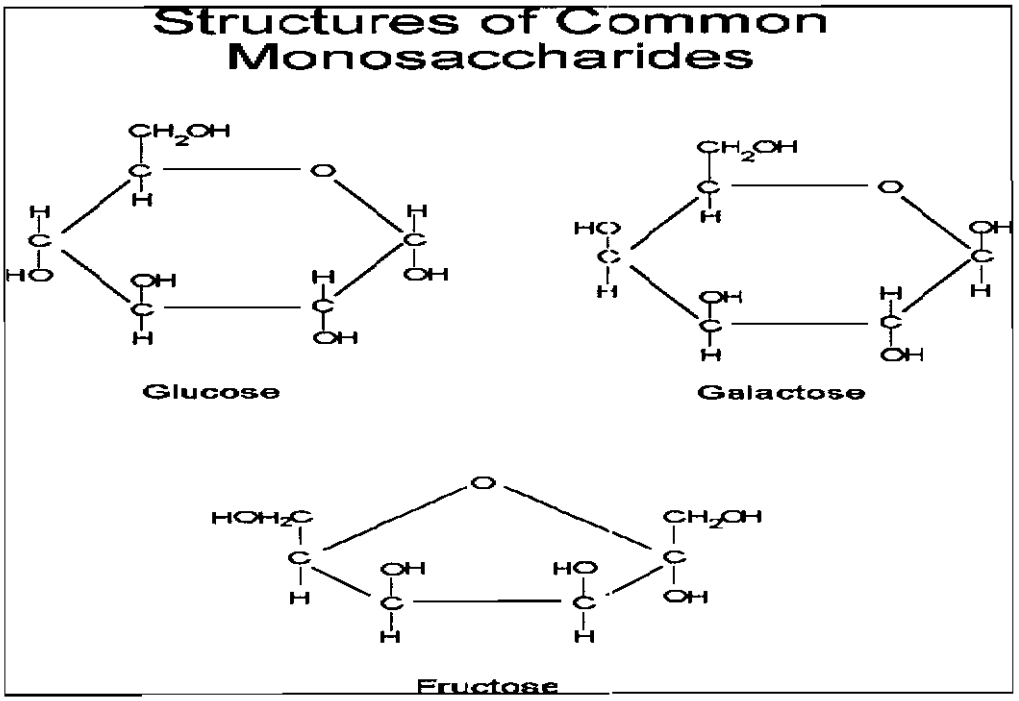
I. Carbohydrates are a good and cheap source of energy which can spear expensive protein for growth
II. Regulate blood glucose
III. Carbohydrates provide dietary fiber that increases the enzyme action on nutrients resulting in good digestion.
IV. Increase taste and palatability of diet

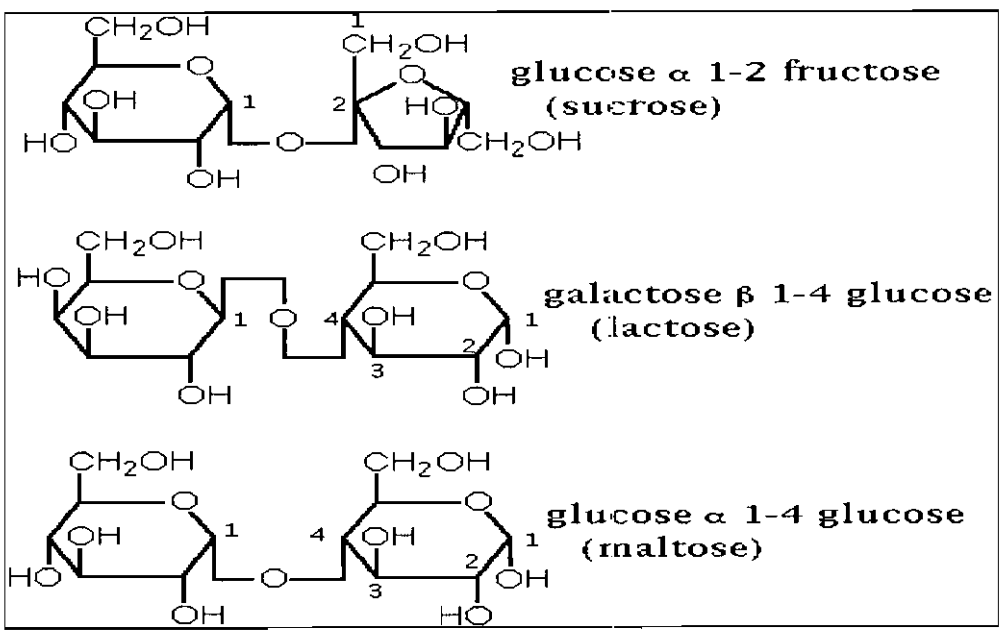
2-2. Properties and structures of carbohydrates (CHO)

Properties of carbohydrates:

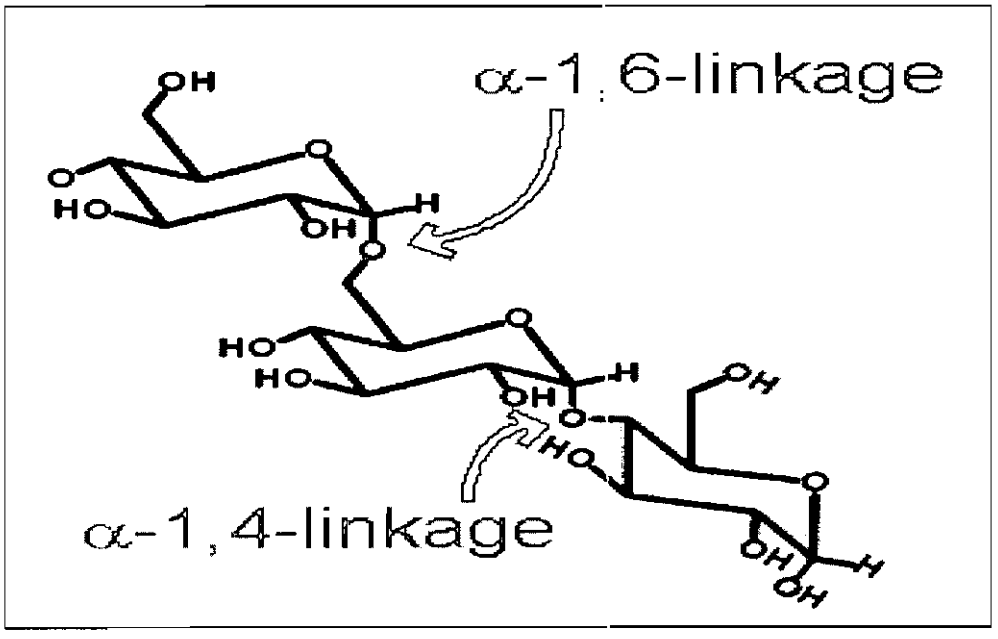
- Carbohydrates are called carbohydrates because they are essentially hydrates of carbon (i.e. they are composed of carbon and water and have a composition of $(CH_2O)_n$.
- The major nutritional role of carbohydrates is to provide energy and digestible carbohydrates provide 4 kilocalories per gram. No single carbohydrate is essential, but carbohydrates do participate in many required functions in the body.
- Central to all metabolic processes.

Structures of carbohydrates:





Disaccharides



Starch

2-3. Essentiality of CHO and new concept of CHO in nutrition

Carbohydrates are found in a wide variety of naturally occurring substances and serve as principal energy sources for the body. It is a cheap source of energy. Maximum fish can not digest carbohydrates because of lacking of cellulose. Warm water fish can utilize carbohydrates to some extent. Therefore, carbohydrates are not essential nutrients for fish. Only a few animals like termites, cows, goats etc are able to digest cellulose. Therefore, carbohydrates are essential nutrients for those animals.

3-1. Definition, Classification and functions, deficiency symptoms and dietary sources of vitamins

Definition of vitamin: Vitamins are groups of organic substances that are required in relatively small amount in the diet of animals for normal growth, maintenance of life and normal reproduction.

Classification of vitamin: Vitamin can be categorized into two grups.

- **Fat-soluble vitamin (stored in the tissue)**

I. **Vitamin A**
II. **Vitamin D**
III. **Vitamin E**
IV. **Vitamin K**

- **Water-soluble vitamin (not stored in the tissue)**

I. **Vitamin B complex (Thiamine, Riboflavin, Panththenic Acid, Nicotinic acid, Pyridoxine, Biotin, Folic acid, Cyanocobalamine)**
II. **Vitamin C (ascorbic acid)**
III. **Choline**
IV. **Insitol**

Functions, deficiency symptoms and dietary sources of vitamins:

Vitamin	Function	Deficiency symptom	Dietary sources
FAT-SOLUBLE VITAMINS			
A (Carotene)	Synthesis of visual pigments	Night blindness and blindness in children	Egg yolk, liver, green and yellow vegetables, fruits
D (Calciferol)	Regulation of calcium metabolism	Rickets (malformation of the bones)	Milk, action of sunlight on the skin
E (Tocopherol)	Antioxidant, protection of cell membranes, reproduction	Fragile RBC, reproductive failure	Vegetable oil
K	Essential for prothrombin formation and blood clotting.	Blood-clotting disorders	Leafy vegetables, intestinal bacteria
WATER-SOLUBLE VITAMINS			
B_1 (Thiamine)	As a coenzyme in energy metabolism, promotes appetite and growth.	Beriberi, neuritis, mental disturbance	Brain, liver, heart, whole grains
B2 (Riboflavin)	Promotes growth, important in carbohydrate and amino acid metabolism	Photophobia, dermatitis	Milk, eggs, liver
Niacin	Constituent of	Pellagra, dermatitis,	Whole grains, liver

	coenzymes, hydrogen transport.	digestive problems	
Pantothenic acid	Component of coenzyme A, required for energy metabolism.	Neuromotor and cardiovascular disorders	Most foods
B_6 (Pyridoxine)	As coenzyme in protein and nitrogen metabolism, RBC formation.	Dermatitis, nervous disorders	Whole grains, liver, fish, kidney
Biotin	Component of several enzyme systems.	Scaly dermatitis, muscle pains, weakness	Egg yolk, intestinal bacteria
Folic acid	Related to B_{12} metabolism.	Anemia	Liver, leafy vegetables, intestinal bacteria
B_{12} (Cobalamin)	Coenzyme in several enzyme system.	Pernicious anemia	Liver, kidney, brain
C (Ascorbic acid)	Collagen formation, formation of intercellular substances of the teeth, bones, and soft tissues.	Scurvy, failure to form collagen	Citrus fruits, green leafy vegetables, tomatoes
Choline	Involved in nerve impulses.	Fatty livers, kidney hemorrhaging	Choline chloride
Inositol	Not known.	Spectacled-eye appearance in rats	Synthetic inositol, all feeds.

3-2. Properties and structures of fat soluble vitamins

General properties of fat soluble vitamin:

- As the name implies, this group of vitamin is fat soluble
- Fat soluble vitamins are primarily stored in the liver and adipose tissue in appreciable quantities and may cause hypervitaminosis thereby
- The fat soluble vitamin do not serve as coenzymes but rather act directly or bind to specific receptors in the cell nucleus to influence gene expression
- Vitamin E acts as antioxidant

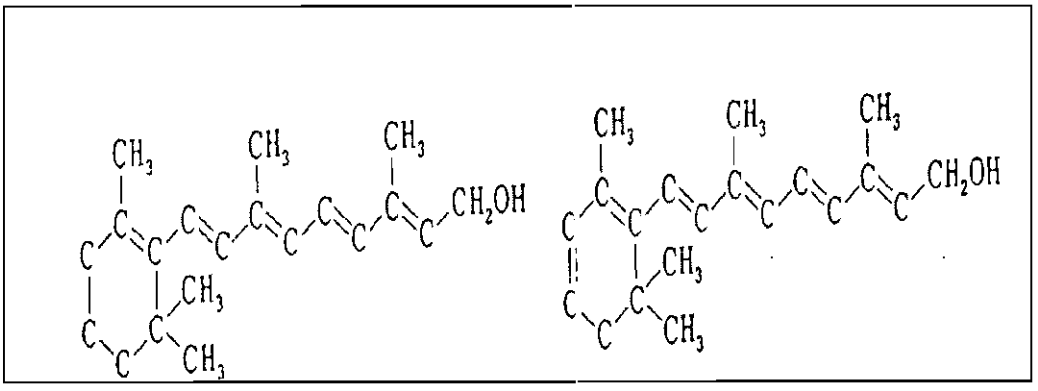
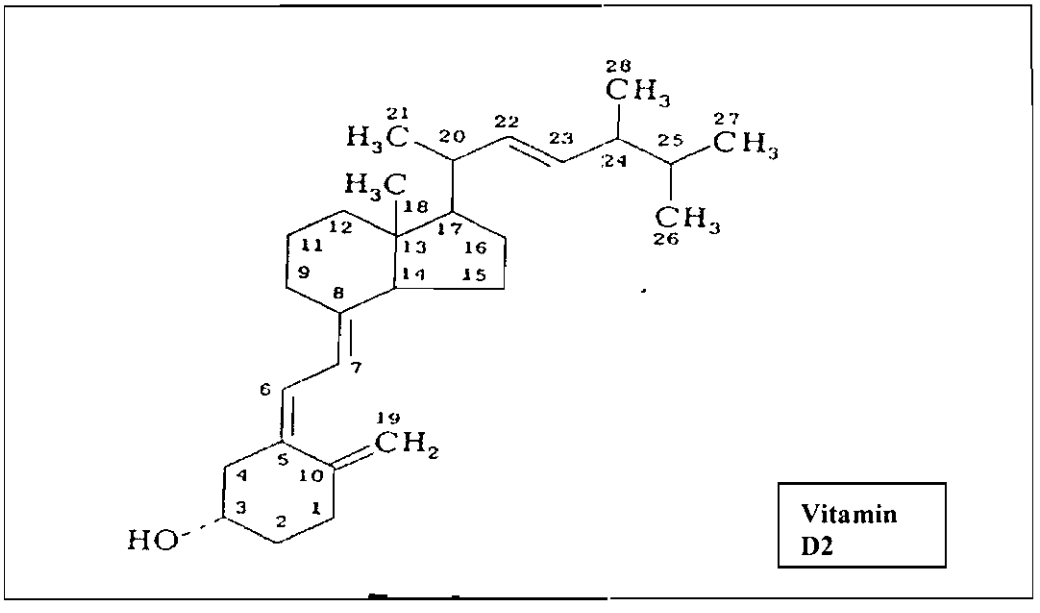
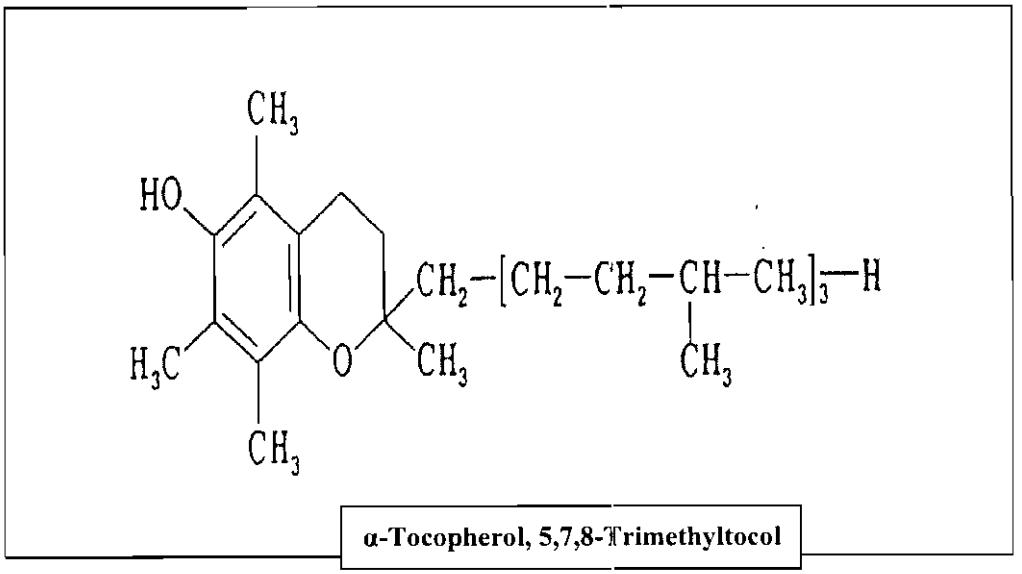
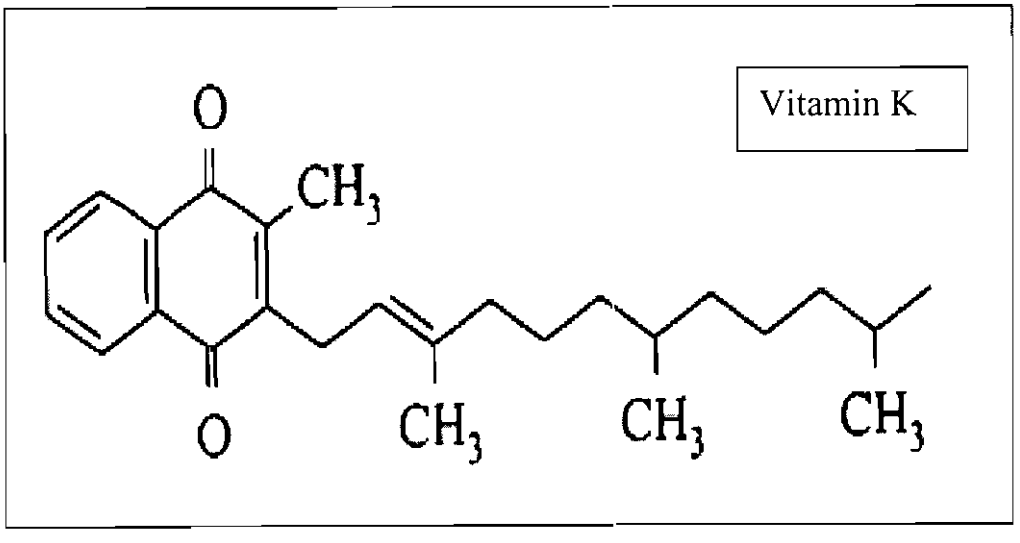


Figure: **Left: all-trans-vitamin A1 (retinol1), Right: all-trans-vitamin A2**





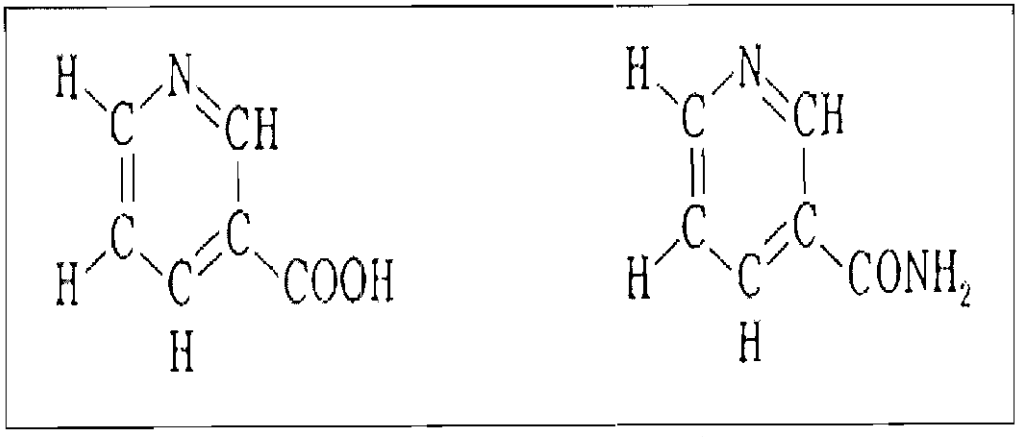
α-Tocopherol, 5,7,8-Trimethyltocol



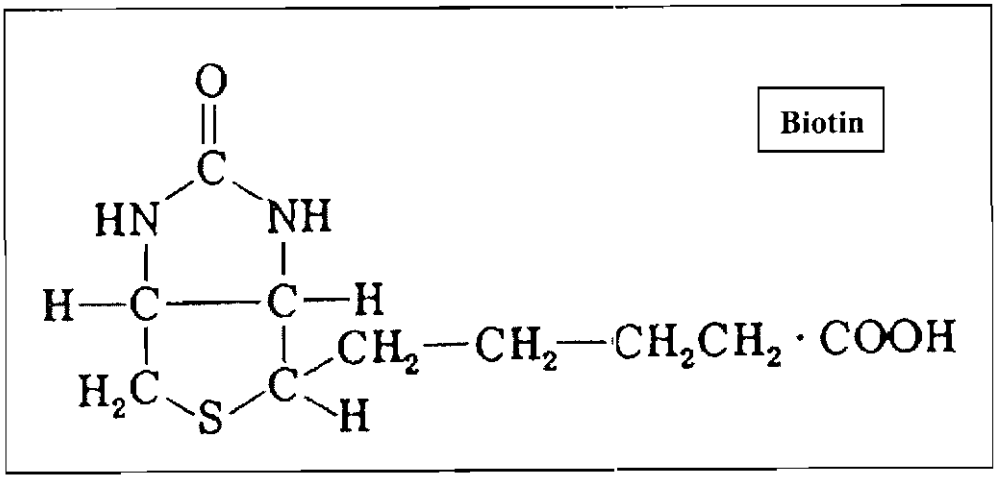
3-3. Properties and structures of water soluble vitamins

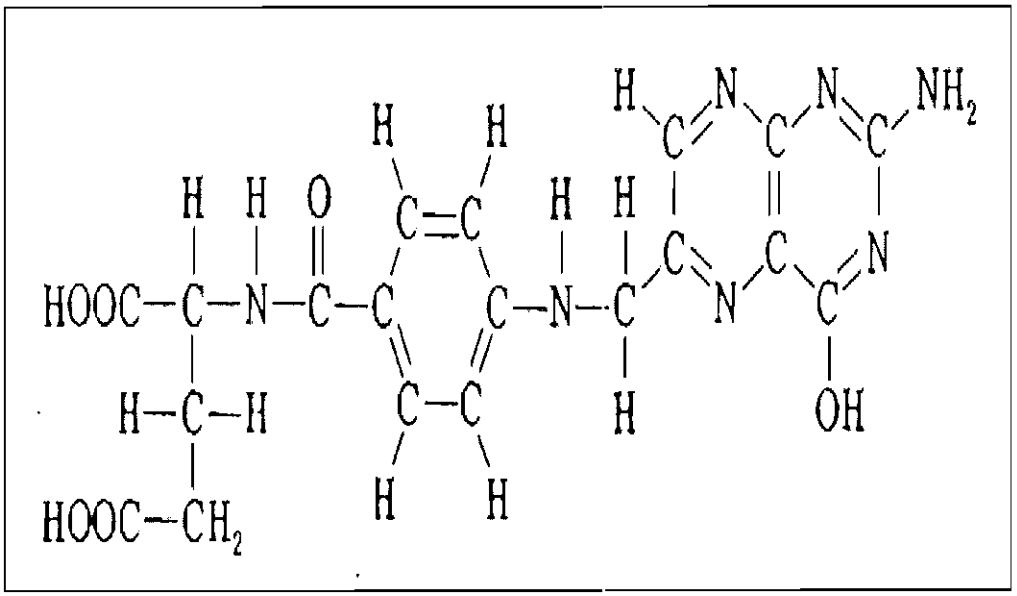
General properties of water soluble vitamin:

- Soluble in water
- Water soluble vitamin can not be stored in the body and readily flashed out through urine; hence deficiency takes place very often
- Vitamin B can be synthesized by microbial fermentation in the digestive tract, especially of ruminants herbivorous nonruminents (horse and rabbit)
- Water soluble vitamins being components of many coenzymes play an important role in a variety of biochemical reactions

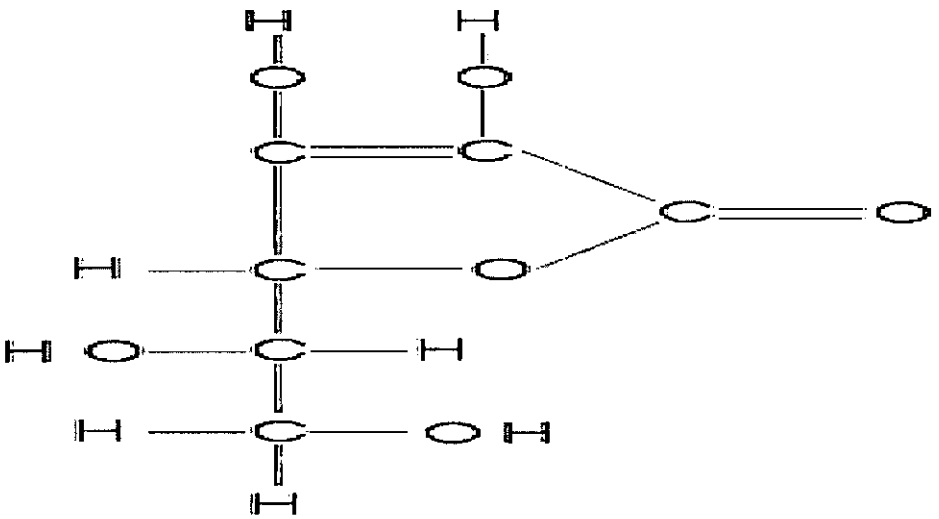


Niacin: nicotinic acid (left) and niacinamide (right)





Folic acid



Vitamin C

4-1. Definition and Function of Minerals

Definition of Minerals

All forms of aquatic animals require inorganic elements or minerals for their normal life processes. Unlike most terrestrial animals, fish have the ability to absorb some inorganic elements not only from their diets but also from their external environment in both freshwater and seawater. Calcium (Ca), magnesium (Mg), sodium (Na), potassium (K), iron (Fe), zinc (Zn), copper (Cu), and selenium (Se) are generally derived from the water to satisfy part of the nutritional requirements of fish, while phosphates and sulfates are more effectively obtained from feed sources. The exchange of ions from the surrounding water across the gills and skin of fish complicates the measurement of mineral requirements. Moreover, many essential elements are required in such small quantities that it is difficult to formulate diets and maintain an environment that is low in minerals to demonstrate a mineral deficiency. An Essential element is that element whose deficient intake produces an impairment of function, and the restoration of physiological levels of the element prevents or relieves the deficiency.

Funtion of Minerals

The main functions of inorganic elements in fish include the formation of skeletal structure, electron transfer, maintenance of colloidal systems (osmotic pressure, viscosity, diffusion) and regulation of acid-base equilibrium. Minerals are also important components of hormones and enzymes, and they activate enzymes. Calcium and phosphorus (P) are required for the formation of the skeletal structures of the body. Sodium, potassium, magnesium, calcium, chloride, phosphates and bicarbonates maintain homeostasis and the acid-base balance. Trace metals such as Fe, Mn, Cu, Co, Zn, Mo, and Se are firmly associated with specific proteins in metalloenzymes, which produce unique catalytic functions. Some minerals, such as calcium, magnesium, and manganese, function as enzyme activators. Iodine, a nonmetal, is necessary for the biosynthesis of thyroid hormones, which in turn affect development and metabolism in all vertebrates.

Currently, 29 of the 90 naturally occurring elements are known to be essential for animal life and they are categorized into macro- and micro elements depending on the quantities required in by fish.

4-2. Major Minerals

Macro elements: These elements are required by the fish in relatively large quantities (grams per kilogram diet). They include Na, K, Ca, P, Mg, Cl and S.

Micro elements: Unlike the macro elements, the micro elements, also called the trace elements, are needed in small quantities (milligrams or micrograms per kilogram diet). Although these elements are needed in small amounts, they are still important in the normal life processes of fish and inadequate intake of the minerals leads to the development of deficiency symptoms. They include Fe, Mn, Cu, Co, Zn, Mo, I, and Se.

Calcium and Phosphorus: Calcium and phosphorus are involved in the development and maintenance of the skeletal system and participate in several physiological processes. Fish scales are an important site of calcium metabolism and deposition. Besides its structural functions, calcium plays an important role in muscle contraction, blood clot formation, nerve impulse transmission, the maintenance of cell integrity and acid-base equilibrium, and activation of several important enzymes.

Phosphorus is an important constituent of nucleic acids and cell membranes, and is directly involved in all energy-producing cellular reactions. Phosphorus also functions in carbohydrate, lipid, and amino acid metabolism, as well as in various metabolic processes involving buffers in body fluids.

Dietary requirements: The calcium requirement of fish is met largely by absorption through gills and skin in freshwater and by drinking seawater. The calcium requirement is affected by the water chemistry, the phosphorus level in the diet, and species differences. Channel catfish and salmonids require 0.45 and 0.34% calcium, respectively in their diets.

Feed is the main source of phosphate for fish because the concentration of phosphate is low in natural waters. The dietary supply of phosphate is more critical than that of calcium because fish must effectively absorb, store, mobilize, and conserve phosphate in both freshwater and seawater environments. Dietary phosphorus requirements of 0.45 and 0.60 % have been reported for channel catfish and salmonids.

Deficiency symptoms: Calcium deficiency has not been detected in carp and catfish in freshwater. The feed ingredient of natural ingredient diets supplies sufficient calcium to meet the requirements of most finfish. Deficiency signs reported in certain finfish include reduced growth, poor feed conversion, anorexia, and reduced bone mineralization.

The main phosphorus deficiency signs in most fish include poor growth, anorexia, poor feed conversion, reduced bone mineralization, skeletal deformity, cranial deformity, curved and enlarged spongy vertebrae and increased visceral fat.

Magnesium: Magnesium is a major intracellular cation that is a necessary cofactor for enzymatic reactions vital to every major metabolic pathway. Magnesium is a cofactor of many enzymes in the body including those involved in cellular respiration and transfer of phosphate between adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP). These enzymes are phosphokinases, thiokinases, phosphatases, pyrophosphatases, and amino acyl synthatases. Extracellular magnesium is vital to normal nerve conduction, muscle function, and bone mineral formation. Magnesium plays an important role in the respiratory adaptation of freshwater fish.

Dietary requirements: The magnesium requirement of fish can be met either from the diet or water. In the marine environment, magnesium supplementation of diet may not be necessary. The magnesium requirements of channel catfish and salmonids are 0.04 and 0.05%, respectively.

Deficiency symptoms: Magnesium deficiency causes reduced growth, anorexia, sluggishness, nephrocalcinosis, convulsions, cataracts, degeneration of muscle fibers and epithelial cells of pyloric cecum and gill filaments, skeletal deformity, reduced bone mineralization, reduced bone, body and serum Mg concentration, and mortality.

Sodium, Potassium and Chlorine: Sodium, potassium and chlorine are the most abundant electrolytes in the body. Sodium and chlorine are the principal cation and anion, respectively, in the extracellular fluid of the body; whereas, potassium is the major monovalent intracellular cation. Chloride ion is the major anion of gastric juice and blood.

Dietary requirements: The abundance of these elements in common feedstuffs used in fish diets means they need not be supplemented in most natural ingredient diets. However, supplementation of these elements has been found necessary in chemically defined diets for certain species.

Deficiency symptoms: Deficiency signs of these elements are difficult to produce because fish readily absorb these elements from the surrounding aquatic medium. Signs of potassium deficiency include anorexia, convulsion, tetany and mortality.

Iron: Iron is an essential element in the cellular respiratory process through its oxidation-reduction activity and electron transfer. It is found in the body mainly as heme compounds (hemoglobin and myoglobin), heme enzymes (cytochromes, catalase, peroxidase, etc), and nonheme compounds (transferring, and iron-containing flavoproteins [ferredoxins, dehydrogenases]).

Dietary Requirements: Feed is the major source of iron for fish because natural waters usually contain low amounts of soluble iron. The iron requirements have been reported for channel catfish and salmonids as 30 and 60 mg/kg, respectively.

Deficiency and toxicity symptoms: Deficiency of iron causes reduced growth and poor feed conversion, hypochromic microcytic anemia, low hematocrit and hemoglobin levels, and reduced plasma Fe and Fe transferrin saturation. The major effects of iron toxicity include reduced growth, increased mortality, diarrhea, and histopathological damage to liver cells.

Copper: Copper is a constituent of many enzymes and is essential for their activities. It is associated with cytochrome *c* oxidase of the electron transport chain in the cell. Other cuproenzymes found in fish tissues include superoxide dismutase, tyrosinase, lysyl oxidase, ceruloplasmin, and dopamine ß-hydroxylase.

Dietary requirements: The dietary requirements for copper in selected fish have been reported. Channel catfish requires 5mg/kg and salmonids require 4mg/kg.

Deficiency and toxicity symptoms: Deficiency signs in certain finfish include reduced growth, cataracts, reduced liver Cu/Zn-superoxide dismutase and heart cytochrome *c* oxidase activity. Fish appear to be more tolerant of copper in the diet than of dissolved copper in the water. Concentrations of 0.8 to 1.0 mg/kg copper per liter as copper sulfate in water are toxic to many species of fish. Toxicity signs include reduced growth and feed efficiency and elevated liver copper levels.

Zinc: Zinc is an integral part of a number of metalloenzymes, including dehydrogenases, aldolases, peptidases, and phosphatases.

Dietary Requirements: Fish accumulate zinc from both water and dietary sources; however, dietary zinc is more efficiently absorbed than water borne zinc. The zinc requirements of channel catfish and salmonids are 20 or 150 mg/kg and 15-30 mg/kg, respectively.

Deficiency and toxicity symptoms: Deficiency of zinc leads to reduced growth, anorexia, short-body dwarfism, cataracts, fin erosion, skin erosion, reduced body zinc, bone zinc, and bone calcium concentrations, low serum zinc level, and mortality. A zinc concentration of 1,000 mg/kg diet resulted in reduced hemoglobin, hematocrit, and hepatic copper concentrations in rainbow trout.
Manganese: Manganese functions either as a cofactor that activates metal-enzyme complexes or as an integral part of certain metalloenzymes in carbohydrate, lipid, and protein metabolism. Many kinases, transferases, hydrolases, and decarboxylases can be activated by either manganese or other divalent cations, such as magnesium, and their activity is not specific for manganese. However, enzymes such as glycosyl transferase are highly specific for manganese activation. Two important manganese metalloenzymes are pyruvate carboxylase and superoxide dismutase.
Dietary requirements: Although the uptake of manganese from water has been demonstrated, it is more efficiently absorbed from feed. The manganese requirements of channel catfish and salmonids are 2.40 and 16 mg/kg, respectively.
Deficiency Symptoms: Reduced growth, loss of equilibrium, short-body dwarfism, cataracts, high mortality, reduced bone and body manganese concentration, poor hatchability of eggs, and abnormal tail growth.

Selenium: Selenium is an integral part of the enzyme glutathione peroxidase. Glutathione peroxidase destroys hydrogen peroxide and fatty acyl hydroperoxides in water and fatty acyl alcohols, respectively, by using reducing equivalents from glutathione, thereby protecting cells and membranes against oxidative damage. The exact physiological role of this enzyme is not clear because catalase and non-Se-dependent glutathione peroxidase also remove hydroperoxides. Selenium (and selenium compounds) also protects cells against the toxicity of heavy metals such as cadmium and mercury.
Dietary requirements: The selenium requirement of fish varies with the form of selenium ingested, polyunsaturated fatty acid and vitamin E content of the diet, and concentration of waterborne selenium. The selenium requirement determined on the basis of optimum growth and maximum plasma glutathione peroxidase activity was estimated to be 0.25 mg/kg for channel catfish and 0.15-0.38 mg/kg for salmonids.
Deficiency symptoms: Reduced growth, anemia, cataracts, muscular dystrophy, exudative diathesis, and reduced glutathione peroxidase activity.
Iodine: Iodine is essential for the biosynthesis of the thyroid hormones, thyroxine and triiodothyronine.
Dietary requirements: Fish obtain iodine from water and from feed sources. The minimum iodine requirement of most fish species has not been established.
Deficiency symptoms: Iodine deficiency causes thyroid hyperplasia.

Mineral requirements in channel catfish and salmonids

Mineral	Dietary requirement	
	Channel catfish	Salmonids
Calcium	0.45 %	0.34 %
Phosphorus	0.45 %	0.60 %
Magnesium	0.04 %	0.05%
Zinc	20 or 150 mg/kg	15-30 mg/kg
Selenium	0.25 mg/kg	0.15-0.38 mg/kg
Manganese	2.40 mg/kg	16 mg/kg
Copper	5 mg/kg	4 mg/kg
Iron	30 mg/kg	60 mg/kg

4-3. Semi-essential Minerals

Unit V

Feeds, Feed stuffs and Feed formulation practice:

1. Feeds and Feed stuffs
2. Feed formulation practice

1-1. Classification of Feeds (FAO)
1-2. International Feed Number(My Book and FAO)
1-3. Quality Control requirements for Fish meal and Fish oil (My Book and FAO)
2-1. Basic concepts of computer feed formulation
2-2. Feed formulation practice I with 2-4 feeds stuffs for flounder(tables)
2-3. Feed formulation practice II
2-4. Feed Formulation practice III

FEEDS AND FEED STUFFS

Semi-intensive and intensive aquaculture practices differ from extensive practices in that an external feed input is required for the well-being of the stock. The feed input constitutes one of the major recurring costs of the operation, its share often increasing in proportion to the increasing intensity of the culture operation. Consequently, it is essential that the principles underlying diet preparation are well understood.

Adequate nutrition is one of the most important factors influencing the ability of cultured organisms to attain the genetic potential for growth, reproduction and longevity. The nutrient requirements vary between species and within species between the different stages of its life-cycle. The main objective and/or aim of diet formulation and preparation is to utilize the knowledge of nutrient requirements, locally available feed ingredients and digestive capacity of the organism for the development of a nutritionally balanced mixture of feedstuffs which will be eaten in adequate amounts to provide optimum production of the cultured organism at an acceptable cost. In practice however, besides nutritional value, cost and availability of ingredients, other considerations have to be taken into account in diet preparation. Such considerations include pelletability of the resulting diet, anti-nutritional factors in the ingredients, and diet acceptability or palatability.

Diet formulation involves the selection and blending of appropriate feed ingredients to produce a diet with the required quantities of essential nutrients. No single ingredient can be expected to meet all the nutrient requirements of a cultured organism. By selecting various ingredients in the correct amounts, a compounded ration which is nutritionally balanced, pelletable, palatable and easy to store and use may be formulated.

Basic information required for feed formulation includes:

- The nutrient requirements of the cultured species
- The feeding habitats of the species
- Local availability, cost and nutrient composition of ingredients
- Ability of the cultured species to utilize nutrients from various ingredients as well as the prepared diet

- The anticipated feed consumption
- Feed additives needed
- Type of feed processing to be employed

DEFINITIONS

- **Additive:** An ingredient or combination of ingredients, other than a premix, added to the basic feed mix or parts thereof to fulfill a specific need. Usually used in micro-quantities and requires careful handling and mixing.

- **Complete Feed:** A nutritionally adequate feed for animals other than man: by specific formula is compounded to be fed as the sole ration and is capable of maintaining life and/or promoting production without any additional substances being consumed except water.

- **Compound Feed:** A mixture of products of vegetable or animal origin in their natural state, fresh or preserved, or products derived from the industrial processing thereof, or organic or inorganic substances, whether or not containing additives, for oral feeding in the form of a complete feed.

- **Concentrate:** A feed used with another to improve the nutritive balance of the total and intended to be further diluted and mixed to produce a supplement or a complete feed.

- **Feed (Feedstuff):** Any substance, whether processed, semi-processed or raw, which is intended for animal consumption.

- **Food:** Any substance, whether processed, semi-processed or raw which is intended for human consumption, including drinks, chewing gum and any substance which has been used in the manufacture, preparation or treatment of 'food' but excluding cosmetics, tobacco and substances used only as drugs.

- **Formula Feed:** Two or more ingredients proportioned, mixed and processed according to specifications.

- **Hazard:** A biological, chemical or physical agent in, or a property of, feed which may have an adverse effect.

- **HACCP:** Hazard analysis critical control point.

- **Ingredient:** A component part or constituent of any combination or mixture making up a (commercial) feed.

- **Medicated feed:** Any feed which contains drug ingredients intended for the treatment or prevention of disease of animals other than man. [N.B. Antibiotics used as growth promoters are considered to be 'feed additives'.

- **Premix:** A uniform mix of one or more micro-ingredients with a diluent and/or carrier. Premixes are used to facilitate uniform dispersion of micro-ingredients in a larger mix or a mixture of additives, or a mixture of one or more additives with substances used as carriers, intended for the manufacture of feed.

- **Straight Feedstuff or Straights:** A vegetable or animal product in its natural stat e, fresh or preserved, and any product derived from the industrial processing thereof, and single organic or inorganic substance, whether or not it contains any additive, inte nded as such for feeding.

- **Supplement:** A feed used with another to improve the nutritive balance or perfor mance of the total and intended to be: (i) fed undiluted as a supplement to other feeds; (ii) offered free choice with other parts of the ration separately available; or (iii) furth er diluted and mixed to produce a complete feed.

FEED INGREDIENTS

Fish feed ingredients are mainly by-products of food processing obtained when high-value food for humans is extracted from a raw material. After extraction of the main products, the by-products are further processed, usually by drying. Some feed ingredients, however, are produced directly from raw materials. A wide variety of ingredients are available for use in fish feeds. These include protein supplements, energy sources, vitamin premixes, mineral premixes, and non-nutritive feed additives.

Feed Nomenclature: The international Feed Vocabulary (IFV) is a system of naming feed ingredients so as to avoid confusion. Through this system, a comprehensive name and number has been assigned to each ingredient using descriptions from one or more of six categories. The categories are

- Origin, including the scientific and common names for specific plants and animals, poultry, fish, cereals, grass, minerals, chemical products, and drugs or other names for nonspecific materials.
- Part fed to animal as affected by processing
- Process(es) and treatment(s) to which the feed ingredient was subjected
- Stages of maturity and development
- Cutting (for forage crops)
- Grade

Using this system, herring meal is described as fish, herring, *Clupea harengus*, meal, mechanically extracted, International Feed Number 5-02-000.

Protein Supplements: Protein supplements are feed ingredients having protein content above 20%. These include:

Animal by-products: These are derived from the meat-packing, poultry processing, and rendering industries. The protein content of these products ranges from 50 to 85%. These meals are good sources of lysine but poor sources of methionine and cystine, which are usually found to be limiting in diet formulations. Animal by-products include meat meal and meat and bone meal, blood meal, feather meal, poultry by-product meal, milk by-products and gelatin.

Fish products and by-products: Fishmeal is the most suitable animal product for incorporation into fish diets. The major fish meals include anchovy, capelin, menhaden, sand eel, herring, whitefish, and salmon. They may be made of whole fish, as is the case with anchovy, capelin, or menhaden, or from processing residue, as is the case with

whiting, pollock, herring, and salmon. Fish meal contains high levels of essential amino acids. The fat content ranges from 4 to 20%, and the ash content is highly variable, ranging from about 11 – 12% in anchovy meal to over 23% in whitefish meals made from filleting wastes. Other by-products include shrimp and crab by-products, fish silage and fish hydrolyzate.

Plant protein sources: Oilseed meals are the most important protein supplements of plant origin. They are produced from the cake remaining after oil has been extracted from oilseeds such as soybeans, cottonseed, canola, peanuts, sunflower seeds and coconuts. Oils may be mechanically expelled or extracted by solvents. They may be decorticated (when the coating is removed before extraction), dehulled (without hull) or undecorticated (hull and coat intact). Some intermediary products between decorticated and undecorticated exist. The composition of oilseed meals varies not only between species of plant but also according to the processing methods employed. Dehulled soybean meal contains 48% while defatted soybean meal contains 44% protein; cottonseed meal has a protein content of 48%; corn and wheat gluten meals contain more than 60% protein while brewer's dried yeast contains 27% protein.

Other protein sources: Another category of protein supplements is sometimes referred to as unconventional feedstuffs. They include single-cell proteins derived from yeast or bacteria grown on waste materials such as paper mill sludge, sewage, crop processing wastes, and methane. The dried products could contain up to 60 – 70% protein. Other unconventional feedstuffs include krill and dried insect meal.

Energy Sources: Basal feeds, or energy feeds, are low-protein, high-energy feed ingredients. The upper limit for protein content of basal feeds is 20%, although most are in the 10 – 17% range. They include fats and oils as well as carbohydrates, although the availability of carbohydrates to fish varies from species to species.

Vitamin Premixes: These are concentrates in which stable forms of essential vitamins are mixed with a carrier, usually a basal feed such as a wheat by-product.

Mineral Premixes: Mineral premixes are concentrates of essential elements that are fortified in practical fish diets to make up for low levels in the formulation or to overcome antagonistic interactions among feed ingredients.

Nonnutritive Feed Additives: These include pellet binders, carotenoid supplements, therapeutants and nonspecific immune stimulants, probiotics, enzyme supplements, hormones, antimicrobial agents, antioxidants, fiber, water, flavorings and palatability enhancers.

FORMS OF FEEDS

Aquafeeds can exist in different forms. However, they are generally classified into three groups based on their moisture contents. These include:

Wet Feeds: Generally, wet feeds are those made from wet ingredients such as trash fish, slaughterhouse waste, undried forage, etc., and contain 50–75% moisture.

Semi-moist Feeds: Semi-moist feeds are made from mixtures of wet and dry raw materials, or from dry ingredients to which water is added. The moisture content of these feeds ranges from about 20 to 30%.

Dry Feeds: Dry feeds are generally made from dry ingredients or from mixtures of dry and moist ingredients. However, even though it may be implied by the name, these feeds are not entirely devoid of moisture, generally containing 7–13% water, depending on the environmental conditions.

TYPES OF FEEDS

Feeds could be formulated based upon the life stage of an animal. Feed formulations can be categorized by the intended function or phase of production in which the feed will be used. Specific feeds can be formulated to increase survival, growth, fish health (nonspecific immunostimulation), fish quality, and body composition or to minimize the amount of nutrients in hatchery or farm effluents (pollution reduction). The main types of feeds according to the intended use are:

First Feed for Fry and Larvae: These include starter feeds (larger than 400μm in diameter) and larval feeds (less than 400μm in diameter).

Fry Feeds: Fry feeds are fed to fry or larvae that are actively feeding and have reached a weight of 0.50-0.75 g.

Conversion and Transition Feeds: Conversion feeds are offered when fish are converting from live food to formulated feed while transition feeds are fed when fish have been fed highly palatable starter feed, but are ready to move to a lower palatability fingerling or grower feed.

Fingerling Feeds: These are feeds for fish weighing 10-100 g. Semimoist pellet sizes of 1.2-2.4 mm cover this range. Dry, compressed feeds are fed as crumbles to fish at the lower end of their weight range and as 2.4-3.00 mm pellets to larger fish.

Grower Feeds: Grower feeds are formulated to promote efficient and economic growth of fish from the fingerling stage up to market size. Formulations for these fish contain less protein and more energy than fingerling formulations. The energy content and protein to energy (P/E) ratio is carefully formulated to ensure that protein retention is high. The majority of feed fed during a production cycle, often more than 90%, is fed during the grower stage and any savings in cost of feed at this phase is of a great significance in minimizing total costs of production.

Broodstock Feeds: Broodstock requires higher nutrients than growers for gonadal development. Protein, essential fatty acids, vitamins and minerals are required in relatively higher quantities at this stage for good fecundity, egg quality and larval performance.

Low-Pollution Feeds: These are produced with the aim of minimizing pollution caused by effluents. Feeds are formulated to contain optimum energy with ingredients of high digestibility for nutrients such as phosphorus.

Product Quality Feeds: Product quality feeds are those that are fed to fish to increase the quality of the product in the market. Formulation adjustments are made to enhance quality attributes such as color, lipid content, fatty acid composition, percentage dress-out, shelf-life, flavor and texture of fish.

FEED FORMULATION TECHNIQUES

Best-Buy Ingredients: Besides other considerations, feed ingredients should be selected in a formulation on the basis of value. If several fish meals are available and suitable for use in a formulation, and the fish meals are equal in protein content and protein availability, then the least expensive one is chosen. However, if the meals differ in protein content or protein availability and price, calculations are required to determine which has the best value.

Simultaneous Equations for Solving Least-Cost Analysis: Once the ingredients are chosen, simultaneous equations can be used to solve simple feed formulations. The process of formulating a fish feed is divided into several steps. The first step is to define the nutrient levels desired in the feed. The second step is to choose ingredients and to list the protein and digestible energy content of each. The third step is to list the ingredients which will be in the feed formulation at fixed levels and to calculate the contribution of each ingredient to the total desired levels of protein and digestible energy in the feed. These values are then added, and the totals subtracted from the desired levels in the finished feed.

Linear Programming: Least-cost formulations can be calculated using computers. The process by which this is done is called linear programming. This involves the simultaneous solution of a series of linear equations. Linear programming is used to calculate the combination and levels of ingredients that provide the desired nutrient content of the diet at the least cost. To do this, the following information must be provided to the computer:

- Nutrient content and digestible or metabolizable energy of ingredients.
- Nutrient requirement of the species for which the feed is intended.
- Unit price of feedstuffs including vitamin and mineral mixtures.
- Any other additives to be used in the feed.
- Minimum and maximum restrictions on the amounts of each ingredient in the feed.

FEED MANUFACTURING

The basic steps in diet manufacture are grinding, mixing, conditioning, pelleting, cooling (or freezing), top-dressing, sacking, storing, and shipping. Ingredients arrive at feed mills either in sacks or in bulk, and in both dry and liquid forms. Ingredients should be examined for condition on arrival, and samples taken for analysis and dated. Drugs and medications must be segregated. Ingredients must be stored to protect against

contamination, moisture, and pest damage. Each ingredient should be used in the order of batch delivery to prevent deterioration associated with long storage.

Grinding: Many ingredients arrive at the feed mills in a coarse form and need to be ground before being used. When ingredient particle size is not uniform and sufficiently small, mixing inefficiencies and blockage of dies may occur during pelleting. Grinding increases the surface area of ingredient particles, thus facilitating mixing and pelleting. Various kinds of grinders reduce particle size by impact (hammer mills, pulverizers, and attrition mills), cutting (rotary cutters, roller mills, and attrition mills), and crushing (roller mills). Different types of particle size reduction equipment vary considerably in initial cost and in operating cost, but the primary factor affecting the choice of equipment should be the range of ingredients that the equipment will be required to grind.

Mixing: Mixing of ingredients is a critical operation in diet manufacture. Inadequate or excessive mixing leads to particle segregation, resulting in a nonhomogeneous blend. Microingredients (vitamins, minerals, drugs and carotenoid pigments) are premixed separately with a diluent, such as wheat flour, before being mixed with other dietary ingredients. The mixing procedure should result in a blend from which all of the pellets contain the same proportion of ingredients as the formulation. Ingredients are generally added to the mixer in a predetermined sequence to ensure adequate mixing. Dry ingredients are first combined, with dry premixes added in the middle of the sequence. After sufficient mixing of dry ingredients, liquid ingredients are added and mixing continues. It is important to know the proper amount of time for mixing with each type of mixer and type of feed blend. Mixing can be done with a batch or continuous mixer. Types of mixers include horizontal ribbon mixers, vertical mixers, Nanta mixers, and turbine mixers. For laboratory use, dough mixers are appropriate.

Conditioning and Expansion: Conditioning is the process of preparing a feed mixture for pelleting and includes thermal and physical processing. Thermal processing is generally accomplished by the addition of steam in a conditioning chamber. The chamber may be pressurized or it may be open to the atmosphere. Conditioning chambers contain agitators that mix and work the feed mixture as steam is added. Besides ensuring that added moisture is thoroughly mixed with the feed mixture, agitators activate some ingredients, such as wheat gluten.

Expansion is a conditioning step that occurs after mixing but before compression pelleting. The process involves steam injection and mixing in a preconditioning chamber, followed by pressure (shear) being applied along a barrel. The mixture is then forced through a narrow gap created by the presence of a cone in a tapered outlet of a chamber. Heat from steam, pressure (shear), and frictional energy generated as the feed mixture squeezes through the gap causes starch gelatinization. As the pressure is lost when the mixture exits the gap, moisture is lost. The mixture is then conveyed through a steam (compression) pelleting system, and the resulting pellets are identical to steam pellets, except for the degree of gelatinization, which also affects the pellet density and the amount of oil that can be top-dressed. Pellets made by this process can be top-dressed to achieve up to 22% total fat.

Pelleting: Pelleting is aimed at converting the homogeneous blend of ingredients into durable particles having physical characteristics that make them suitable for feeding. There are many ways to produce feed pellets. Each feed manufacturer has developed particular methods to increase the quality or decrease the cost of their feed. These include the following:

Compressed pelleting: Compressed pelleting, also known as steam pelleting, is a process which forces a feed mixture, which has been exposed to dry steam for about 5-25 seconds to increase the temperature to about 85 ^{0}C and the moisture to about 16%, through holes in a metal die by the action of a roller located inside the die. The combination of heat, moisture, and pressure forms the mixture into a compressed pellet (bulk density, 0.5-0.6 g/cm^3) in which gelatinization of the starch occurs. As the pellets emerge from the outside surface of the die, they are cut off by a stationary, adjustable knife to the desired length.

Pellet quality is influenced by several factors, including fat level, moisture, and humidity. The fat level of the mixture should be no lower than 2-3% to lubricate the holes in the die and to reduce dustiness and no higher than 8-10% to avoid excessive die lubrication causing insufficient compression of the feed mixture. The moisture level also affects the quality of the pellets, as insufficient moisture results in production of dry and crumbly pellets while excessive moisture leads to the production of soft pellets due to insufficient compression.

Extruded Dry Pelleting: Extruded pellets are made in the same general way as compressed pellets, but the use of different dies and physical conditions results in a very different product. This process is more versatile than compression pelleting, thereby allowing for the control of pellet density, which affects buoyancy in water, and for the addition of very high lipid levels. However, this process is more expensive than steam pelleting. In this process the temperature of the feed mixture is increased to 125-150 ^{0}C in a pressurized conditioning chamber and the moisture content is increased to 20-24%. The density of the pellets is typically 0.25-0.3 g/cm^3.

UPC pelleting: The universal pellet cooker combines some aspects of compressed pelleting and cooking extrusion. The pelleting equipment resembles cooking-extrusion equipment. Less steam and water are added during preconditioning than in cooking extrusion, resulting in a feed mixture with 16-18% moisture. Pellets of bulk density ranging from 400 to 600 g/liter can be produced using this system.

Cold pellet extrusion: This process is employed in manufacture of semimoist pellets. By varying the moisture content and by carefully selecting ingredients with binding activity, a stable, durable moist pellet can be produced. In contrast to other methods of pellet formation, no thermal activity is involved in cold extrusion.

Cooling and drying: Immediately after manufacturing, the pellets are cooled and dried by passing them through a cooler-dryer. Cool air is blown through the pellets, which are spread thinly on a moving belt in a horizontal cooler. Vertical coolers drop hot pellets through a cooling tower. The pellets are hot when leaving the pellet mill and this heat facilitates drying. For laboratory-scale pelleting, cooling and drying can be accomplished by spreading the pellets and blowing air over them with a fan.

The dry pellets have moisture content of about 10%. Extruded pellets contain more moisture than compressed pellets and therefore, need to be heated to reduce moisture to 10% or less.

Crumbling and screening: Dry pellets are crumbled by passing the cooled, dried pellets through the corrugated rollers of a crumbler. The objective in crumbling is to maintain a high rate of production of smaller particles without producing excessive amounts of fines. This is controlled by adjusting the speed of the rollers and the distance between them. The crumbles are screened to appropriate feed sizes using a shaking, vibrating screen.

Coating (Top-Dressing): The total amount of lipid required in feed formulations cannot be included in the mixture being pelleted, due to processing limitations, except in the case of twin-screw extrusion. High-lipid mixtures simply do not pellet; the lipid lubricates the mixture, limiting compression. Hence, lipid is added after pelleting, either by conveying the pellets through a continuous oil spraying chamber or in batch system. Very high pellet lipid levels require that the oil is added to pellets in a vacuum chamber. The benefits of top-dressing include:

- High lipid levels can be achieved.
- Heat-sensitive substances, such as enzymes, pigments, and vitamins, can be added after pelleting.
- Feed palatability can be increased by coating with palatability-enhancing substances.

Shipping and Storage: After pellets have been cooled, dried, screened, and top-dressed, they can be transferred to bins for storage and bagging. Feed can be delivered to farms in bulk by truck, transferred by auger to storage bins at the farm, and fed. Bagged or sacked feed is generally placed on pallets, wrapped in plastic, and shipped. This prevents excessive feed handling, which can break pellets and tear bags. The maximum storage time for pelleted feeds after manufacture depends on many factors, including the type of manufacturing, formulation, use of antimicrobial compounds, storage conditions, and source and quality of dietary fat. Generally, dry feeds should be used within 90 days, while moist feeds should be used within 60 days.

INGREDIENT AND DIET EVALUATION

Ingredients and finished diets can be evaluated by a variety of chemical and biological tests. These tests are used to check on the accuracy of the manufacturing process arriving at a finished feed of the desired composition (quality control), to measure nutrient loss during manufacture and storage, to predict the nutritional value of a particular formulation, to detect oxidative rancidity, and to measure the nutritional or feeding value of a formulation. The best method to evaluate a diet formulation is to feed it to a group of fish and compare their growth to that of fish fed a standardized diet for which the nutritional value is known. However, this is rarely practicable; hence, chemical analysis is usually employed in place of this method. The following methods are employed in evaluation of ingredients and diets.

Proximate Analysis: Water, crude protein, ether extract, crude fiber, nitrogen-free extract and ash.

Nutrient Analysis: This involves direct measurement of the individual essential nutrients in feeds by chemical analysis. The nutrients include amino acids, fatty acids, minerals, and vitamins.

Chemical Tests: This includes protein and lipid quality evaluations.
Protein quality evaluation: Pepsin digestibility, available lysine, total volatile nitrogen (TVN) and ammonia-nitrogen, and biogenic amines.
Lipid quality evaluation: hydrolytic and oxidative rancidity (peroxide value, thiobarbituric acid-reactive substances (TBARS) test, anisidine value, Kries test, Schall oven test)

Chemical Score and Indispensable Amino Acid Index (IAAI):

$$\text{Chemical score} = \frac{\text{g limiting amino acid in test protein}}{\text{g amino acid in whole-egg protein}} \times 100$$

$$\text{IAAI} = \frac{\text{ARG (TP)}}{\text{ARG (WEP)}} + \frac{\text{HIS (TP)}}{\text{HIS (WEP)}} + \ldots + \frac{\text{VAL (TP)}}{\text{VAL (WEP)}} \times 100$$

Biological Evaluation:
General methods: Weight gain, specific growth rate, feed conversion ratio, apparent digestibility coefficient, and carcass deposition or nutrient retention.
Protein and amino acid quality evaluation methods: Biological value, protein efficiency ratio (PER), net protein utilization (NPU), and protein retention.

$$\text{PER} = \frac{\text{weight gain (g)}}{\text{Protein fed (g; dry weight basis)}}$$

Unit VI

Aquaculture and New area of research :

1. Direction of future aquaculture
2. Fish nutrition research review
    (1) Develop new species diets
    (2) Develop low pollution diet
    (3) New techniques for research

1-2 Current status and future prospects of World Aquaculture
2-1 Develop new species diets (Korean rockfish)
2-2 Develop low pollution diets (Flounder)
2-3-1 New techniques for research (Operation Techniques in trout)
2-3-2