Effects of B Vitamins on Protein Utilization from Rice-Legume Dietaries by the Growing Rat[1]

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Several reports point out the beneficial effects of higher dietary supplements of B vitamins on the efficiency of protein utilization. The literature on the subject has been extensively reviewed in recent communications from this laboratory (Marfatia and Sreenivasan, '60a, b; Fatterpaker et al., '60; Wagle and Sreenivasan, '61). These studies have shown that B vitamins at optimal levels improve utilization of protein in growing rats fed diets containing single and mixed proteins. These observations have been extended to demonstrate the effects due to vitamin B_{12} and folic acid, and the impairment in growth and nitrogen retention in rats arising from split-feeding of protein and carbohydrate components of the diet, as well as from intermittent feeding of high and low protein diets. These could be offset by using an optimal supplement of B vitamins in the diet. Studies with protein-starved rats have demonstrated that the turn-over of liver and plasma constituents is influenced by the levels of certain B vitamins, more specifically of folic acid and vitamin B_{12} (Wagle and Sreenivasan, '60).

Our knowledge of the optimal requirements for essential amino acids for laboratory animals and man and of the amino acid content of dietary constituents makes it possible to supplement diets with amino acids deficient in them (Sure, '53, '57a; Westerman et al., '57). Likewise, small supplements of a high quality animal protein have been used frequently in diets to make up the nutritional deficiencies in low quality proteins (Sure, '57b, c; Westerman et al., '57; Deshpande et al., '55).

The object of the present investigation was to assess the effects of B vitamin, amino acid and high quality protein supplementation of a poor quality diet typical of that consumed by low income groups of South India. This diet, conforming to the average local practice, included rice and a legume (*Cajanus indicus*) as major constituents with leafy and nonleafy vegetables, milk and also condiments in quantities as normally used. The cereal: legume proportions were blended to give diets containing two protein levels.

EXPERIMENTAL AND RESULTS

In all experiments, Wistar rats of either sex were housed individually in raised mesh-bottom cages, with access to the diets in excess quantities and in scatter-proof cups.

Two isocaloric diets containing 6.8% (diet 1) and 10.2% (diet 2) of total protein as determined by analyses for total nitrogen by the Kjeldahl method were prepared. The composition of these diets is presented in table 1. Rice and legume (*Cajanus indicus*) contributed 5.22 and 1.15% respectively, of protein to diet 1 and 2.62 and 6.90% protein to diet 2, the rest of the protein being derived from the other constituents of the diet. The vegetables were macerated and blended with the other ingredients. The diets were steamed for one-half hour in an autoclave and later dried under vacuum at 70°C for 5 hours. The dried material was pulverized and stored in the cold for use.

The B vitamins, where provided, were added at one of two levels (table 1), which corresponded to the optimal (high) and minimal (low) requirements of these vitamins for the growing rat (Marfatia and Sreenivasan, '60a).

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TABLE 1

Composition of the rice-legume diets[1]

Components	Diet 1	Diet 2
	gm	*gm*
Milled white rice	74.0	36.5
Legume (*Cajanus indicus*)	5.0	30.0
Fresh milk	5.0	10.0
Nonleafy vegetables[2]	8.0	10.0
Leafy vegetables[3]	2.0	5.0
Spice mixture[4]	2.5	2.5
Arachis oil[5]	2.5	5.0
Sucrose or vitaminized sucrose	1.0	1.0

Vitaminized sucrose (B vitamins per gm of sucrose)

	High level	Low level
	mg	*mg*
Thiamine·HCl	0.3	0.015
Riboflavin	0.4	0.015
Pyridoxine·HCl	0.3	0.015
Ca pantothenate	1.0	0.1
Niacin	2.0	0.1
Biotin	0.05	—
Folic acid	0.1	0.003
Vitamin B_{12}	0.015	—
Inositol	20.0	5.0
Choline Cl	20.0	5.0

[1] Where vitaminized sucrose was added, addition of B vitamins was made either at high or low level as indicated.

[2] Consisting of edible portions of eggplant (*solanum melogena*) and okra (*Hibiscus esculentus*) in equal proportion.

[3] Consisting of amaranth (*Amaranthus gangeticus*) and spinach (*spinacia oleracea*) in equal proportion.

[4] Consisting of (parts): sodium chloride, 1; tamarind fruit pulp, 0.7; pepper, 0.1; chillies, 0.1; turmeric, 0.1; and cinnamon, 0.1; with additions of calcium (as calcium lactate) and iron (as ferrous ammonium sulphate) at levels of 50 and 2 mg, respectively, per 100 gm of the spice mixture.

[5] Fat-soluble vitamins: α-tocopherol, menadione, vitamin A acetate and vitamin D (calciferol) were added to the diet in amounts (per 100 gm diet) of 5, 1, 0.31 and 0.0045 mg, respectively.

The diets were analyzed for their essential amino acid content. One-gram samples were hydrolyzed in 25-ml sealed glass bulbs with 10 ml of 6 N HCl for 10 hours at 15 p.s.i. The hydrolysates on cooling were filtered and adjusted to pH 7.0.

The essential amino acids, other than tryptophan, were assayed microbiologically according to Barton-Wright ('52). Tryptophan was determined in enzymic digest of the diets. Samples equivalent to one gram of protein were suspended in 50 ml of water and, after adjustment of the pH to 1.5 with 6 N HCl, the suspensions were hydrolyzed with 10 mg of pepsin[3] at 37° for 18 hours. Hydrolysis was continued with 10 mg of trypsin[4] at 40° for 24 hours after addition of 3 gm of KH_2PO_4 and adjustment of pH to 8.4 The hydrolysate was finally neutralized and washed with ether to remove indole, anthranilic acid, etc., made to volume and filtered. Assay of tryptophan in the filtrate was according to Barton-Wright ('52). The results are tabulated with the amino acid requirement for normal growth in the rat (Rose, '37) (table 2). Diet 1 is deficient in all the essential amino acids except arginine and valine, whereas diet 2 is limiting in only three amino acids — methionine, lysine and tryptophan.

Data on food consumption and body weight were recorded during the 8-week period. The liver analyses for total and nonprotein nitrogen and total lipids, as well as determinations of total and nonprotein nitrogen in blood plasma, were carried out at the end of each experimental period according to methods detailed elsewhere (Marfatia and Sreenivasan, '60a). The protein-free filtrates of plasma were used in one experiment for the assay of free essential amino acids; the filtrates were treated for this purpose in the same manner as were the diet hydrolysates.

TABLE 2

Essential amino acid composition of the rice-legume diets[1]

Amino acid[2]	Diet 1	Diet 2	Requirement for normal growth (Rose, '37)
	Percentages of diet		
Arginine	0.44	0.8	0.2
Histidine	0.19	0.38	0.4
Isoleucine	0.38	0.66	0.5
Leucine	0.52	0.78	0.8
Lysine	0.35	0.8	1.0
Methionine	0.3	0.4	0.6
Phenylalanine	0.48	0.96	0.7
Threonine	0.3	0.64	0.5
Tryptophan	0.08	0.08	0.2
Valine	0.9	1.3	0.7

[1] *Leuconostoc mesenteroids* P 60 was used for the assay of lysine, histidine, methionine and phenylalanine; *Lactobacillus arabinosus* 17-5 was used for the assay of tryptohan, leucine, isoleucine and valine; arginine and threonine were assayed using *Streptococcus faecalis*.

[2] L-forms of lysine, histidine and arginine and DL-forms of methionine, tryptophan, threonine, phenylalanine, leucine, isoleucine and valine were used as standards. Values expressed are, however, in terms of the L-amino acid (50% in the case of racemic standards).

[3] Nutritional Biochemicals Corporation, Cleveland.

[4] See footnote 3.

Supplementation with B vitamins. Weanling male rats weighing 40 to 45 gm were used in a pilot experiment. The animals were divided into 4 groups of 6 rats each and fed diets 1 and 2 supplemented with B vitamins at the high level or unsupplemented. Although significant increases in the growth rate and in the efficiency of protein utilization were obtained by increasing the level of dietary protein, or, at the high protein level, by increasing the B-vitamin concentration, the diets failed to support good growth in general (table 3). This effect could be attributed to the poor over-all quality of the protein in these diets; and since the amino acid requirements of immature animals for optimal growth are more exacting than those of young adult ones for normal growth (Hegsted and Worcester, '47), the above experiment was repeated using male rats of 90- to 95-gm initial weight 6 to a group. Data were also obtained in this experiment on total and nonprotein nitrogen in the liver and nonprotein nitrogen and free essential amino acids in blood plasma. Results on protein efficiency ratio and liver constituents are included in table 3. A marked over-all improvement in growth may be seen from this table. Increased protein concentration and vitamin supplementation considerably enhanced both growth and protein efficiency ratio. There was also a striking increase in the liver total nitrogen concentration as a result of increasing the dietary protein level; vitamin supplementation produced relatively small gains. The liver nonprotein nitrogen concentration was not influenced by the dietary protein level but was reduced as the diet was fortified with B vitamins. There was a decrease in nonprotein nitrogen in the rats fed the high vitamin diets, the effect being more at the high protein level (table 4). These changes were associated with significant reductions in plasma levels of histidine, methionine and threonine. It may be noted that, as a result of increasing the dietary protein, the concentration of every amino acid in the plasma was also increased when the diets were not supplemented with B vitamins; in the presence of B vitamins, reductions in methionine and threonine contrations were observed despite raising the protein level.

TABLE 3

Effect of diet and B-vitamin supplementation on growth and protein efficiency in weanling and young adult rats

Group		Weanling rats			Young adult rats				
								Liver[4]	
Protein	B vitamin[1]	Gain in weight[2]	Food intake	PER[3]	Gain in weight	Food intake	PER	Total nitrogen	Non-protein nitrogen
%		*gm*	*gm/day*		*gm*	*gm/day*		*mg/gm*	*mg/gm*
6.8	−	11.0 ± 5.5[5]	3.8 ± 0.2	0.77 ± 0.04	18.2 ± 3.1	5.8 ± 0.2	0.82 ± 0.04	21.3 ± 0.7	2.5 ± 0.02
6.8	+	16.7 ± 4.9	4.1 ± 0.3	1.07 ± 0.02	28.3 ± 3.6	6.7 ± 0.4	1.11 ± 0.05	25.1 ± 0.8	2.3 ± 0.05
10.2	−	23.8 ± 3.4	3.9 ± 0.2	1.06 ± 0.05	34.1 ± 2.6	5.5 ± 0.3	1.08 ± 0.02	29.4 ± 0.7	2.4 ± 0.03
10.2	+	36.8 ± 3.4	4.3 ± 0.3	1.50 ± 0.07	50.2 ± 5.9	6.4 ± 0.5	1.37 ± 0.04	32.7 ± 0.6	2.2 ± 0.04

[1] The diets were with (+) or without (−) a supplement of B vitamins at the high level as indicated in table 1.
[2] Mean weight gains per rat over a period of 8 weeks.
[3] Protein efficiency ratio, expressed as grams gain per gram of protein consumed.
[4] Values are expressed on the fresh weight of tissue.
[5] Average of 6 rats/group ± standard error of the mean.

TABLE 4

Effect of diet and B-vitamin supplementation on plasma nonprotein nitrogen and free amino acids[1]

Group[2]		Non-protein nitrogen	Arginine	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenylalanine	Threonine	Tryptophan	Valine
Protein	B vitamins[3]											
%		*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
6.8	−	27.0 ± 1.2[4]	2.41 ± 0.06	0.54 ± 0.01	0.58 ± 0.09	1.62 ± 0.13	5.21 ± 0.07	0.40 ± 0.07	1.41 ± 0.15	4.07 ± 0.42	1.18 ± 0.12	2.32 ± 0.25
6.8	+	24.8 ± 0.8	2.32 ± 0.08	0.44 ± 0.04	0.61 ± 0.11	1.56 ± 0.03	5.34 ± 0.91	0.32 ± 0.04	1.43 ± 0.21	3.82 ± 0.38	1.21 ± 0.16	2.46 ± 0.31
10.2	−	27.2 ± 1.4	2.72 ± 0.14	0.74 ± 0.05	0.72 ± 0.06	1.82 ± 0.16	6.28 ± 0.61	0.65 ± 0.02	2.12 ± 0.36	4.59 ± 0.21	1.51 ± 0.18	2.91 ± 0.24
10.2	+	21.2 ± 0.9	2.61 ± 0.21	0.59 ± 0.01	0.68 ± 0.08	1.61 ± 0.19	6.04 ± 0.58	0.21 ± 0.04	2.21 ± 0.36	2.82 ± 0.21	1.48 ± 0.16	3.04 ± 0.23

[1] For standards, L- forms of lysine, histidine and arginine and DL-forms of methionine, tryptophan, threonine, phenylalanine, leucine, isoleucine and valine were used. Values expressed are, however, in terms of the L- amino acids (50% of the racemic standards).

[2] Each group consisted of 6 young adult rats.

[3] Diets were supplemented with (+) or without (−) the B vitamins at the high level as given in table 1.

[4] Mean value ± standard error of mean.

Diet 1 was eliminated from subsequent experiments owing to its general poor quality, diet 2 serving as the basal ration in these studies.

Amino acid supplementation and B vitamins. Female rats weighing 90 to 95 gm were divided into 6 groups of 6 rats each. One group was fed the basal 10.2% protein ration (diet 2) without additional supplementation of B vitamins. Another group subsisting on this ration received supplements of L-lysine (0.2% of diet), DL-methionine (0.4%) and DL-tryptophan (0.24%). The remaining 4 groups subsisted on the above rations as supplemented with B vitamins at high or low levels (table 1). Data were obtained on protein efficiency ratio and on liver content of total and nonprotein nitrogen and total lipids (table 5).

The efficiency of protein utilization was improved to a greater extent with amino acid supplementation than with the B-vitamin supplement. The animals fed the amino acid-supplemented diet were further benefited by incorporation of B vitamins in the diet. The gains in liver total nitrogen due to B vitamins alone were again small, whereas with amino acid supplementation, these were appreciable. A further increase in liver nitrogen content was obtained when the diet was supplemented with both amino acids and B vitamins at a high level. The nonprotein nitrogen constitutents were apparently not significantly affected by enriching the diet with amino acids. However, with even a low level of B vitamins added to the enriched diet, there was a marked reduction in liver nonprotein nitrogen. With both high and low level supplements of B vitamins the change in nonprotein nitrogen was of the same order. There was a small but significant decrease in liver lipids when the basal ration was supplemented with B vitamins, the change being of equal magnitude at high- or low-level supplementation. A marked decrease in lipids was brought about by amino acid supplementation and, still further, when additional B vitamins were provided at the high level.

Egg albumen supplementation and B vitamins. In another set of experiments, egg albumen replaced 2% of the total legume proteins (6.9%) in the basal ration. The basal ration (diet 2), and also the modified ration, were supplemented with B vitamins at the two levels, high and low. Four groups of male rats, with 6 rats of approximately 95-gm average weight per group, subsisted on these rations for 8 weeks.

The results indicate a marked increase in the efficiency of protein utilization following egg albumen substitution (table 6). The magnitude of the increase in the protein efficiency ratio obtained by raising the dietary B-vitamin level was, however, somewhat lower in the animals receiving egg albumen than in the control animals. These results are substantiated by data on the liver constituents. The B-vitamin level had no significant influence on these constituents in the modified ration containing egg albumen. On the other hand, there were significant reductions in liver nonprotein nitrogen and liver lipids when the vitamin level in the basal ration was increased; also there was a small but significant increase in liver total nitrogen. With egg albumen there was also a marked decrease in liver lipids and in liver nonprotein nitrogen and improvement in protein nitrogen content of the tissue.

DISCUSSION

The improvements in growth rate obtained with B-vitamin supplements might indicate that the basal diet was lacking in one or more of them; however, no syndromes of any B-vitamin deficiency were manifest. The adult rat can, it is known, survive in a reasonably healthy state on very low amounts of certain B vitamins (Miller and Baumann, '44). On the other hand, increased intakes of B vitamins have been shown to result in improved growth (Sure and Romans, '48; Sure, '50; Marfatia and Sreenivasan, '60b). The observed increase in protein nitrogen and decrease in nonprotein nitrogen constituents in the liver with a higher intake of B vitamins are again indicative of a more efficient protein utilization. Vitamin supplementation also lowers concentrations of plasma nonprotein nitrogen and certain free amino acids. Similar observations (Charkey et al., '50, '54) with chicks in relation to vitamin B_{12} have been interpreted to suggest a function for the vitamin in anabolic processes, probably involving channeling of amino

TABLE 5

Effect of B-vitamin and amino acid supplementation of rice-legume diet at 10.2% of protein on growth and liver composition[1]

Supplements[2]			Gains in weight	Food intake	Protein efficiency ratio[3]	Liver		
B vitamins (low level)	B vitamins (high level)	Lysine, methionine and tryptophan				Total nitrogen	Non-protein nitrogen	Total lipids
			gm	gm/day		mg/gm fresh weight		
–	–	–	36.5 ± 4.1[4]	6.0 ± 0.5	1.06 ± 0.05	25.5 ± 0.4	2.6 ± 0.04	129.6 ± 2.7
+	–	–	42.3 ± 4.3	6.2 ± 0.6	1.19 ± 0.01	26.3 ± 0.5	2.4 ± 0.05	122.1 ± 2.9
–	+	–	52.0 ± 3.3	6.5 ± 0.2	1.40 ± 0.02	27.4 ± 0.6	2.3 ± 0.06	123.8 ± 3.1
–	–	+	58.5 ± 5.6	7.1 ± 0.4	1.44 ± 0.004	29.5 ± 0.5	2.5 ± 0.01	99.8 ± 2.6
+	–	+	72.2 ± 3.2	8.3 ± 0.4	1.52 ± 0.03	30.8 ± 0.6	2.2 ± 0.03	102.7 ± 4.2
–	+	+	83.0 ± 5.6	8.0 ± 0.2	1.82 ± 0.05	32.2 ± 0.5	2.1 ± 0.02	95.0 ± 1.8

[1] Data obtained over a period of 8 weeks with 6 young adult rats per group.

[2] Supplements were made (+) or not (–) to the rice-legume diet at 10.2% of protein. The levels of B vitamins were as indicated in table 1. Amino acids, L-lysine, DL-methionine and DL-tryptophan were added at 0.2, 0.4 and 0.24% of diet, respectively.

[3] As defined in table 3.

[4] Mean values and their standard errors.

TABLE 6

Effects of B-vitamin and egg albumen supplementation of the rice-legume diet at 10.2% of protein on growth and liver composition

Supplements[1]	Weight gained in 8 weeks	Food intake	Protein efficiency ratio[2]	Liver		
				Total nitrogen	Non-protein nitrogen	Total lipids
	gm	gm/day		mg/gm fresh weight		
B vitamins (low level)	45.0 ± 1.2[3]	7.6 ± 0.34	1.04 ± 0.03	28.2 ± 0.52	3.2 ± 0.12	95.3 ± 1.5
B vitamins (high level)	63.6 ± 3.7	7.8 ± 0.49	1.43 ± 0.05	29.3 ± 0.44	2.9 ± 0.21	84.0 ± 1.7
Egg albumen + B vitamins (low level)	72.6 ± 3.5	7.5 ± 0.26	1.66 ± 0.02	29.8 ± 0.74	2.7 ± 0.10	77.6 ± 1.9
Egg albumen + B vitamins (high level)	91.5 ± 5.2	8.2 ± 0.28	1.95 ± 0.08	31.5 ± 0.30	2.8 ± 0.13	76.8 ± 1.7

[1] The basal rice-legume diet at 10.2% of protein was supplemented with B vitamins either at low or high level as indicated in table 1. Egg albumen addition was at 2% of protein at the expense of an equal amount of legume protein.

[2] As defined in table 3.

[3] Mean value of 6 animals/group ± standard error of mean.

acids into tissue protein synthesis. In chicks, an effect of dietary vitamins in lowering certain plasma amino acids, while raising others, has been reported by Richardson et al. ('53). The decrease in plasma concentrations of histidine, threonine and methionine with the vitamin supplementation would imply either that the vitamins have a direct functional significance in the metabolism of these amino acids or that a general imbalance of amino acids, reflected in plasma concentrations, is offset, at least to some degree, by higher intake of the B vitamins; it is possible that both causes may be simultaneously operating.

The improvement in growth resulting from supplementation of the diet with its deficient amino acids occurred as expected. Similar nutritional improvements have been observed by others with rice diets (Pecora and Hundley, '51; Pecora, '53; Harper et al., '55). The observed effects of the B vitamins in the presence of the deficient amino acids could mean that for the B vitamins to function, a certain adequacy of dietary amino acids is essential. Probably one aspect of this function is the better utilization of histidine, methionine and threonine as suggested by decreased plasma levels of these amino acids in the previous experiment.

Accumulation of fat in the liver has been suggested as due to the presence of amino acids in the diet in proportions that cannot be utilized by the animal for protein synthesis, the unutilized amino acids contributing to lipogenesis (Harper et al., '55). Partial deficiencies of lysine and threonine have been characterized by such liver fat accumulation (Harper et al., '53; Singal et al., '53). Hence, correction of amino acid deficiencies in the diet may be expected to result in the observed lowering of liver fat. The B vitamins at both high and low levels seem to have relatively little influence on liver lipids. Sarett and Perlzweig ('43) also observed that B-vitamin supplementation of a low protein diet had little influence on liver lipids.

The increased growth obtained with partial substitution of egg albumen for legume protein in the rice legume diet is obviously the result of an improvement in protein quality. The capacity of a protein to promote growth depends essentially upon the adequacy with which the quantity and proportions of the amino acids made available by digestion and absorption match the quantity and proportions needed for tissue synthesis and repair. Similar responses in growth have been observed by other workers (Harper et al., '55) using rice diets supplemented with small amounts of animal protein. In fact, supplementation with animal protein was much superior to that with essential amino acids, the latter often causing imbalances (Deshpande et al., '55). Kik ('56) has demonstrated that fish flour in small quantities added to a rice diet is beneficial for growth. Carpenter and his associates ('57) also reported that fish flours make effective supplements to cereal diets.

The increased growth obtained with the optimal level of B vitamins would indicate the inadequacy of dietary intakes of vitamins when the diets are supplemented with minimal quantities of vitamins. With addition of egg albumen and consequent improvement in the over-all protein quality, the effects due to vitamins were less pronounced. Similar observations have been reported earlier from this laboratory using wheat flour-gluten and wheat flour-egg albumen diets (Marfatia and Sreenivasan, '60).

SUMMARY

The rice-legume diets typical of those consumed by a large section of low-income groups in India were prepared to contain 6.8 and 10.2% of protein and fed to rats for 8 weeks.

Although the diets failed to support good growth in weanling rats, growth was appreciable with young adult rats, and was further improved by B-vitamin supplementation. There were significant reductions in plasma levels of histidine, methionine and threonine in the B-vitamin supplemented group. The amino acid analysis of the diets indicated that the 6.8% protein diet was deficient in amino acids other than arginine and valine, whereas the 10.2% protein diet was deficient in methionine, lysine and tryptophan.

Supplementation of the 10.2% protein diet with the deficient amino acids improved the efficiency of protein utilization and additional B vitamins at high or low levels further enhanced the effect. Amino

acid supplementation of the diet also resulted in a greater decrease in liver lipids than occurred with the B vitamins.

Incorporating egg albumen at a 2% level resulted in marked gains in weight. The effects of B-vitamin supplementation were more pronounced with the basal diet than with the diet supplemented with egg albumen. These results were discussed in terms of amino acid requirements and amino acid-vitamin relationships.

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