Solubility, Amino Acid Composition, and Biological Evaluation of Proteins Isolated from Leguminous Seeds

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Preliminary determination of the nutritional constituents of 19 wild leguminous seeds suggested their possible inclusion in animal nutrition. Since the whole seeds proved unpalatable as well as toxic, it was thought that their proteins might be extracted and used instead. Accordingly, simple and effective methods were standardized for isolation of proteins by subjecting defatted *Phaseolus* seeds to extraction studies. Distribution of total nitrogen and dispersion of nitrogenous constituents in the seeds were also studied. Proteins of considerable purity are best extracted with sodium chloride solution (5%, w./v.), with subsequent dialysis of the extract. Proteins were isolated from four varieties of *Phaseolus* seeds and their protein efficiency ratio and biological value evaluated by animal experiments with and without supplementing diets with the missing essential amino acids.

Since legumes constitute the chief source of proteins in the Indian diet (Barker, 1943; Bressani *et al.*, 1954), many leguminous plants are cultivated on a large scale throughout the country. Many wild and uncultivated plants also grow luxuriantly because of favorable soil and climate conditions (Lugg and Weller, 1945; Subramanian *et al.*, 1952). Faced with protein malnutrition caused by acute food shortage, researchers subjected some of these wild seeds to chemical analysis (Pant and Bishnoi, 1967; Pant and Kapur, 1963a; Pant *et al.*, 1968) and indicated that they were fairly good sources of proteins. However, nutritional experiments on albino rats fed whole seed meals at a 10% protein level proved them unpalatable and in some cases toxic. Therefore, repeating the feeding experiments was proposed, replacing the whole seed meals with isolated seed proteins, cornstarch, and adequate quantities of fats, carbohydrates, vitamins, and minerals. However, first, it was thought desirable to confirm the nutritional efficiency of the globulin type of proteins of edible seed origin as growth promoters in animals. Accordingly, after standardizing of simple and effective methods for the extraction and isolation of seed proteins, globulin-type proteins of considerable purity were isolated from four varieties of *Phaseolus* seeds. The isolated proteins were evaluated for their total α-amino and essential amino acids, protein efficiency ratio, and biological value. The distribution of total nitrogen and the dispersion of nitrogenous constituents in the seeds, by employing different salt solutions at various pH, were also studied.

MATERIALS AND METHODS

The wild leguminous seeds were collected and identified, whereas all the *Phaseolus* seeds were bought locally from the bazaar, powdered to 100-mesh in a hand grinder, and defatted with petroleum ether (b.p. 60° to 80° C.). Moisture, ash, minerals, ether extractives, crude protein, and total soluble carbohydrate were determined by methods previously described (Pant and Bishnoi, 1967; Pant and Kapur, 1963a).

All chemicals used were British Drug Houses analytical reagents. Soluble sugars were determined qualitatively by the paper partition chromatographic technique of Consden *et al.* (1944) as described by Partridge (1946). Defatted seed meal (ca. 1 gram) was repeatedly extracted (7 to 8 times) with ethanol (10 ml., 80%, v./v.), and the clear supernatant was evaporated to dryness. The residue was washed twice with petroleum ether and then dissolved in water (0.5 ml.). The extract (30 to 40 μl.) was employed for chromatography, using the solvent systems described by Pant and Tulsiani (1968).

Fractionation of proteins from seeds was effected by the solubility method of Mitchell (1948). The defatted powder (ca. 12 grams) was successively extracted with water, sodium chloride solution (5% w./v.), ethanol (75% v./v.), and sodium hydroxide (0.25% w./v.) until the extracts were negative to the buret test. Nonprotein nitrogen (NPN) was estimated by employing trichloroacetic acid (2.5% w./v.) as the protein precipitant. The detailed scheme of extraction and fractionation is represented schematically in Figure 1.

Seed proteins were extracted with eight different solvents, (copper sulfate, potassium chloride, bromide, iodide, and sulfate, and sodium chloride, sulfate, and bicarbonate) at 0.25, 0.50, and 1.00*N*. The defatted seed meals (ca. 4 grams) were extracted in duplicate with each of the extractants in 250-ml. Erlenmeyer flasks by shaking for 2 hours in a mechanical shaker and then centrifuging for 15 minutes at 2500 r.p.m. The pH of the clear supernatant was measured in a Leeds & Northrup pH meter and nitrogen was determined in 5 ml. of each extract. From the total nitrogen of the defatted seed meals the per cent of nitrogen extracted was calculated.

The effect of pH variation on the dissolution of proteins was studied by extracting the defatted seed powder (ca. 1 gram) in an Erlenmeyer flask with 25 ml. each of hydrochloric acid and sodium hydroxide solutions of known pH varying from 0.2 to 10.0, shaking the mixture vigorously in a shaker for 2 hours, and then spinning. Nitrogen was estimated in the clear supernatant and from it the percentage of nitrogen extracted from the total nitrogen of the seed meal at different pH was calculated.

The isolated albumin and globulin fractions of proteins were purified by the method of Esh and De (1960), and their purity was tested by paper electrophoresis on an LKB 3276 equipment employing Carl Schleicher and Schull No.

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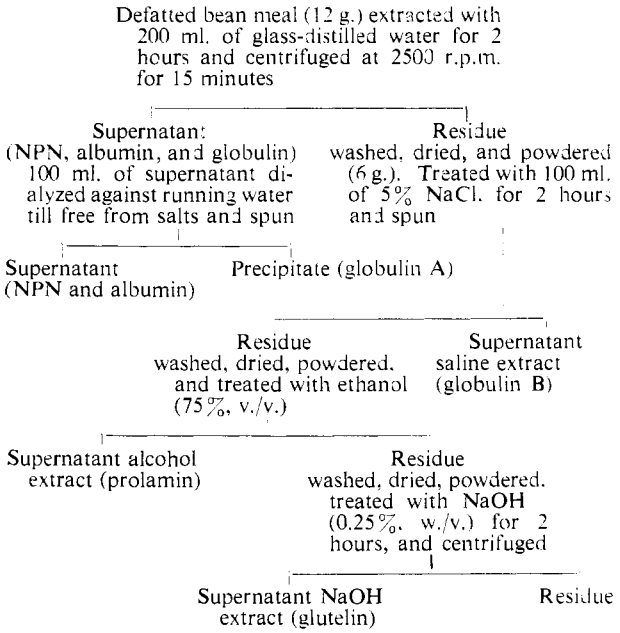

Figure 1. Fractionation chart

2043 B (120 grams per sq. meter, 40 × 410 meters) filter paper strips.

Qualitative amino acid analysis of the isolated seed protein hydrolyzates was carried out by paper partition chromatography on Whatman No. 1 filter paper sheets and their quantitative estimations were made by the elution method of Rosen (1957). Tryptophan was estimated in the unhydrolyzed isolated protein samples by the method of Inglis and Leaver (1964).

Preparation of Protein Hydrolyzates. Accurately weighed purified proteins (20 to 40 mg.) were hydrolyzed in sealed tubes at 100° to 110° C. for 6 to 8 hours with hydrochloric acid (2 ml., 6*N*). The acidic hydrolyzates, negative to the buret test, were repeatedly distilled in vacuo until acid-free to pH 4 to 5 and subjected to chromatographic analysis. The two-dimensional technique of Datta *et al.* (1950) with phenol (80% w./v.)–NH_3 and butan-1-ol–acetic acid–water (4:1:5, v./v.) as developing solvents was employed. Tryptophan was identified in the protein hydrolyzates prepared by refluxing the proteins (50 mg.) with sodium hydroxide (2 ml., 5*N*) for 6 to 8 hours. The chromatograms after development were sprayed with ninhydrin (0.1% w./v.) in 1-butanol. Other specific spray reagents (Pant and Agrawal, 1965) were also used to confirm the identity of different amino acids.

Quantitative Estimation of Amino Acids. Known volumes (2 to 3 μl.) of protein hydrolyzates were applied on Whatman No. 1 filter paper sheets (30 × 30 cm.) in triplicate and dried well after development. One was sprayed with ninhydrin (0.1% w./v.) in butanol to predetermine the location of the different amino acids. This provided guidance, and the different amino acids from the other unsprayed chromatograms were cut carefully and dropped in stoppered glass tubes, to which were then added distilled water (1 ml.), acetate-cyanide buffer (0.5 ml.), and ninhydrin (0.5 ml., 0.5% w./v.) in methyl Cellosolve. The tubes were heated in a water bath at 100° C. for 15 minutes and diluted by the addition of a 2-propanol–water mixture (5 ml., 1 to 1, v./v.). The contents of the tubes were thoroughly mixed and cooled to room temperature (23°), and the color density was measured in a colorimeter at 570 mμ for all amino acids except proline, which was measured at 440 mμ along with a blank and standard glycine solution. Determinations were made in triplicate, and the amount of each amino acid was calculated in terms of glycine.

The biological values of the isolated purified globulin fractions of the *Phaseolus* seeds as well as their protein efficiency ratios were determined by animal experiments on 12 albino rats per group, 4 to 5 weeks old and weighing between 45 and 50 grams. A nitrogen balance sheet method similar to that employed by Chick *et al.* (1935) and Martin and Robinson (1922) and the rat growth method of Osborne *et al.* (1919) as modified by Swaminathan (1937) were followed.

Diets. A nitrogen-free diet containing practically no nitrogen was prepared from starch which was made adequate with respect to other dietary essentials, fats, minerals, and vitamins. Experimental diets were prepared at the 10% protein level, the starch mixed in the nitrogen-free diet being replaced by appropriate weighed quantities of the isolated seed proteins.

To study the quality of proteins from defatted seed powders by animal nutrition experiments, lots of 100 grams of powder were mechanically shaken with sodium chloride solution (1 liter, 5%, w./v.) in large bottles for 2 hours at room temperature (25–27° C.). The extract, after centrifugation at 2500 r.p.m. for 15 minutes, was dialyzed against running water for 24 hours. The precipitated protein was centrifuged, redissolved in the minimum quantity of sodium chloride solution (15 to 20 ml., 5%, w./v.), spun, and reprecipitated by dialysis to effect purification. The solid obtained after centrifugation was twice washed with ethanol, once with acetone, and finally with diethyl ether and dried at room temperature (25–27°).

Experimental diets were prepared by mixing weighed quantities in shallow aluminum pans (2 inches high and 2 inches in diameter) with sufficient water (5 ml. per gram of diet), well stirred, and autoclaved at 10 p.s.i. pressure for 15 minutes. After cooling, weighed quantities of vitamin mixture and shark liver oil (5 drops per rat twice a week) were added to the pans, well stirred, and kept in the cages.

The leftover diets were individually collected daily in tared beakers bearing the numbers of the rat cages, dried in an oven at 100° C. to constant weight, and taken into consideration while calculating the daily food intake of each rat.

RESULTS AND DISCUSSION

Table I shows that some wild inedible seeds contain appreciable percentages of crude proteins (18 to 47) suggesting their possible inclusion in animal nutrition.

Analysis indicates that the four edible *Phaseolus* seeds are good sources of proteins and soluble carbohydrates (Table II). Qualitative analysis revealed the presence of glucose and sucrose in the four *Phaseolus* seeds. Although stachyose has been detected in lupins and soybean (Pigman, 1957) and raffinose in *Phaseolus aconitifolius* (Pant and Tulsiani, 1968), the investigation did not reveal the presence of either sugar.

Table I. Chemical Composition of Some Wild Leguminous Seeds

(Dry weight basis)

Seed	Moisture, G./100 G.	Ash, G./100 G.	Fat (Ether Extractives), G./100 G.	Crude Protein, G./100 G., N × 6.25
Acacia arabica	8.83	4.71	3.32	26.42
Acacia melanoxylon	9.82	4.91	5.54	39.71
Albizzia richardiana	12.45	3.83	7.40	40.00
Bauhinia purpurea	6.95	3.76	16.58	27.18
Cassia glauca	6.65	4.07	7.78	18.25
Cassia obtusifolia	9.93	5.21	5.22	23.90
Cassia occidentalis	9.98	4.66	5.00	35.17
Cassia siamea	13.70	3.38	6.92	21.81
Crotalaria medicaginea	11.89	3.31	3.40	47.46
Crotalaria juncea	10.25	3.15	2.84	33.43
Delonix regia (red flowered)	6.04	3.80	5.03	22.19
Delonix regia (vellow flow red)	5.80	4.70	6.05	27.75
Dolichos biflora	10.58	3.86	2.26	21.35
Erythrina indica	9.04	4.60	15.73	23.08
Glycine hispida	7.90	5.27	21.12	46.82
Mucuna pruriens	7.29	3.87	8.96	29.32
Pongamia pinnata	5.50	3.00	37.50	19.62
Prosopis juliflora	10.94	3.79	4.50	39.25
Sesbania grandiflora	6.25	4.50	7.36	36.55

Table II. Chemical Composition of *Phaseolus* Seeds

(Expressed as percentage on dry weight basis)

Constituent	*Phaseolus vulgaris* Pink Rajmah	*Phaseolus vulgaris* Red Rajmah	*Phaseolus vulgaris* Bakla	*Phaseolus mungo*, Urd
Moisture, %	11.89	12.90	9.90	11.20
Ash, %	3.74	4.35	3.81	3.41
Fat, % (ether extractives)	1.59	1.80	2.35	1.69
Crude protein, N% × 6.25	27.78	28.91	35.10	28.81
Total soluble carbohydrates, %	6.90	6.27	6.18	6.92
Crude fiber, %	1.7	1.5	1.15	3.21
Minerals				
Calcium, mg./100 g.	139	121	188	131
Phosphorus, mg./100 g.	476	351	431	395
Iron, mg./100 g.	8.1	6.3	9.8	7.1

Table III indicates that successive extraction of seed meals with water and sodium chloride solubilizes 74 to 82% of the total nitrogen, which consists of albumin, globulin, and nonprotein nitrogen of combined origin. Globulin contributes the major fraction of total proteins and accounts for 56 to 61% of the total nitrogen. Prolamine forms a small fraction (1 to 3%), whereas nonprotein nitrogen amounts to 10 to 15%, and 4 to 9% remains unextracted in the residue.

Electrophoresis of the albumin and globulin fractions of the four seed meals employing three buffers (acetate, pH 5.0; citrate, pH 6.0; and phosphate, pH 8.0) gave only one band. Although electrophoresis under high voltage should bring about better resolution, the crystalline appearance of the globulin type of isolated proteins under the microscope tempts one to certify the purity of the proteins. This is in conformity with the results of previous authors (Esh and De, 1960; Tawde and Cama, 1960) who claim the homogeneity of seed proteins isolated in the manner described above.

Extraction of proteins by different salt solutions at various concentrations (Table IV) confirms the findings of Smith *et al.* (1938, 1959) and Evans and Kerr (1963). Observation of the inhibiting influence of the divalent Cu^{+2} ions on the solubility of proteins is also in accord with the findings of Smith *et al.* (1938) with soybean proteins.

The effect of pH variation (0.2 to 10.0) on extraction of proteins was studied with hydrochloric acid and sodium hydroxide solutions (Figure 2.) The minimum percentage of total nitrogen extracted in the four *Phaseolus* seeds was between pH 2.1 and 3.38 when only 15 to 22% of the total nitrogen was extracted, mainly albumin and nonprotein nitrogen. Change of pH on either side (1.0 to 1.1 and above 7) solubilized a higher percentage of nitrogen. The maximum percentages extracted on the acidic as well as the alkaline side are approximately similar (about 80%) in all the three *Phaseolus vulgaris* seeds. However, in the case of *Phaseolus mungo* only about 35% of the total nitrogen went into solution on the alkaline side; this could possibly be attributed to the specific nature of *Phaseolus mungo* globulins. To study whether the percentage difference of nitrogen extraction was due to the pH of the extractants, seed meals were extracted with sodium chloride solution (2% w./v.), the pH of which was altered (0.2 to 10.0) by the addition of hydrochloric acid and sodium hydroxide solutions. Points of minimum and maximum extraction of nitrogenous constituents were approximately at the same pH; only the percentage of total nitrogen extracted was different (Figure 3).

Figure 2 suggests possible methods for isolating proteins from dry beans. The proteins could be extracted with hydrochloric acid solution at the pH solubilizing the maximum percentage of nitrogenous components and then changing to the pH of minimum extraction, or extracted with solvents at the alkaline pH (7.5) of maximum extrac-

Table III. Distribution of Total Nitrogen in *Phaseolus* Seeds

Protein Fraction	*Phaseolus vulgaris*			*Phaseolus mungo*
	Pink Rajmah	Red Rajmah	Bakla	
Water-soluble (alb. + globulin + NPN)	66.6 ± 3.12	56.2 ± 3.60	72.4 ± 4.10	33.7 ± 2.05
Albumin	5.5 ± 1.35	6.1 ± 0.85	7.2 ± 1.20	4.1 ± 1.05
Globulin A	43.3 ± 1.65	40.2 ± 1.85	51.3 ± 2.35	15.8 ± 2.50
5% NaCl-soluble (globulin B)	15.5 ± 1.23	20.8 ± 2.10	9.6 ± 1.05	39.9 ± 4.05
Total globulin	58.8	61.0	60.9	55.7
NPN (nonprotein nitrogen)	15.3 ± 1.10	10.1 ± 0.75	13.3 ± 1.20	13.8 ± 1.30
75% ethanol-soluble (prolamin)	1.5 ± 0.31	Traces	2.2 ± 0.15	3.1 ± 0.20
0.25% NaOH-soluble (glutelin)	12.1 ± 0.70	13.8 ± 0.80	8.6 ± 0.60	17.6 ± 1.35
Residue	3.9 ± 0.41	8.8 ± 0.90	6.7 ± 1.05	5.2 ± 0.85

Table IV. Extraction of *Phaseolus* Seed Proteins by Salt Solutions at Different Concentrations

(Mean of duplicate determinations)

Salt	Seed	Concentration					
		0.25*N*		0.50*N*		1.0*N*	
		% total N extracted	Resultant pH of extract	% total N extracted	Resultant pH of extract	% total N extracted	Resultant pH of extract
$CuSO_4$	1	52.8	3.45	54.5	3.42	54.9	3.38
	2	50.7	3.42	53.3	3.37	56.4	3.38
	3	51.2	3.42	57.1	3.37	57.8	3.32
	4	48.1	3.45	56.8	3.38	58.2	3.31
NaCl	1	68.1	6.02	66.3	5.96	66.1	5.85
	2	78.2	6.06	76.9	6.02	75.3	5.96
	3	76.7	5.98	77.3	5.92	77.8	5.90
	4	62.1	6.10	63.4	6.01	63.8	5.94
KCl	1	61.1	6.05	60.1	5.98	58.9	5.95
	2	81.5	6.12	81.9	6.10	81.6	6.05
	3	74.2	6.08	74.8	6.02	75.2	5.95
	4	63.9	6.14	65.3	6.04	66.4	6.03
KBr	1	62.1	5.95	61.2	5.93	60.0	5.90
	2	83.6	6.02	83.0	6.00	81.6	5.92
	3	80.4	6.08	78.7	6.01	76.3	5.95
	4	73.4	6.10	73.0	6.06	72.4	6.00
KI	1	64.0	6.09	63.0	6.03	62.9	5.96
	2	84.1	6.07	83.3	6.07	81.5	6.05
	3	83.3	6.09	80.1	6.03	78.1	5.98
	4	75.1	6.09	75.0	6.03	74.2	5.96
$NaHCO_3$	1	78.6	8.16	77.2	8.20	77.1	8.24
	2	88.3	8.32	87.3	8.38	86.1	8.40
	3	83.1	8.33	82.6	8.35	82.2	8.40
	4	76.1	8.32	75.3	8.38	74.7	8.42
Na_2SO_4	1	65.1	5.83	64.4	5.73	64.2	5.72
	2	80.2	6.00	78.2	5.93	77.2	5.90
	3	73.1	6.00	70.3	5.95	75.3	5.92
	4	67.8	6.02	67.3	5.95	65.3	5.93
K_2SO_4	1	64.8	5.93	63.5	5.12	62.8	5.62
	2	83.2	6.14	82.6	6.10	80.1	6.04
	3	76.3	6.16	77.2	6.10	77.4	6.06
	4	69.0	6.18	68.9	6.12	68.4	6.06

tion and then changing it to that of minimum extraction. The third method is to extract the bean meal with sodium bicarbonate at a distinctly alkaline pH and then precipitate the proteins by adjusting the pH to that of minimum extraction by the addition of hydrochloric acid. Although extraction at higher pH (8 to 10) solubilizes a higher percentage of nitrogenous components in most cases, this does not hold true for *Phaseolus mungo*.

Most of the industrially purified soybean meal proteins are isolated by alkaline extraction (Smith *et al.*, 1938, 1952, 1959), and treating protein fractions with strong acidic and alkaline solutions decreases their nutritive value. In view

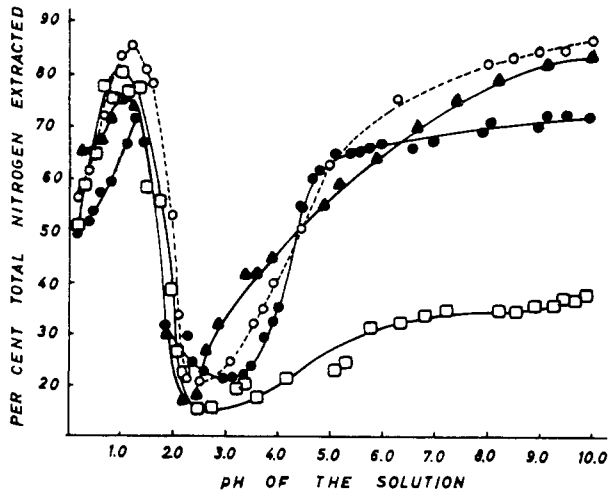

Figure 2. Extraction of nitrogenous constituents from defatted seed meals with HCl and NaOH solutions at different pH

● Pink Rajmah ○ Bakla
▲ Red Rajmah □ *Phaseolus mungo*

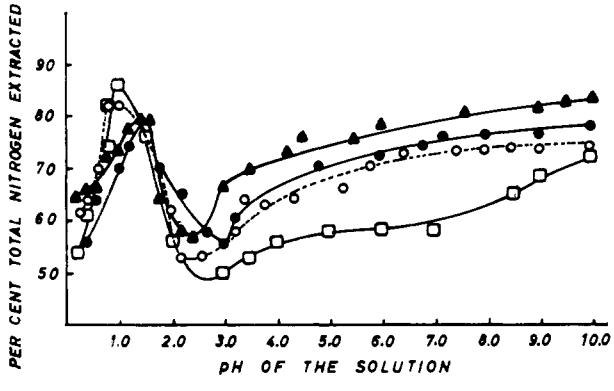

Figure 3. Extraction of nitrogenous constituents from defatted seed meals with NaCl solution (2% w./v.) containing HCl and NaOH at different pH

● Pink Rajmah ○ Bakla
▲ Red Rajmah □ *Phaseolus mungo*

of this, the best and simplest method for isolating proteins from dry seeds would be to extract them with sodium chloride solution and precipitate them by subsequent dialysis of the extract. The proteins thus obtained seem to be of high purity, since they contain less nonprotein material than the samples isolated by the other methods suggested.

Table V, which gives the comparative essential amino acid patterns of the four isolated *Phaseolus* seed albumins and globulins, indicates that the globulins of seeds 2 and 3 are complete proteins with respect to the amino acids essential for animal nutrition. On the other hand, globulins of seeds 1 and 4 reveal only eight essential amino acids.

Thus all the four *Phaseolus* seed globulins examined could be considered as efficient sources of proteins from the nutritional point of view, if those that are incomplete are supplemented with the missing essential amino acids.

The inclusion in animal diets of the isolated albumins, although nutritionally as adequate as the globulins with respect to amino acid composition, would not be economic, since their percentage in the total seed protein is too small (4 to 7).

Although the amino acid composition of the albumin and globulin isolated from different samples of seed would be expected to be similar in composition, they vary significantly, especially with respect to methionine, tryptophan, and the growth factor, lysine. While no specific reason for this variation can be put forward, it is presumed that the maturity and age of the seeds, soil, and genetic and other environmental factors contribute to the chemical composition of the seeds. This necessarily means that although belonging to the "globulin" and "albumin" types of proteins, they do not possess identical amino acid composition qualitatively, much less quantitatively.

Tables VI and VII present the protein efficiency ratio and biological value of the different isolated *Phaseolus* globulins. Growth experiments with young rats revealed that the maximum growth, which is at par with casein diet F, was obtained in animals fed on the isolated protein of *Phaseolus vulgaris* (Red Rajmah), the gain in weight being 44.1 ± 3.8 grams during 5 weeks against 48.1 ± 3.0 grams with diet F. The protein of *Phaseolus vulgaris* (Bakla) registered an increase of 29.6 ± 4.2 grams during the same period. The body weight of experimental animals fed on proteins of the other two varieties of *Phaseolus* seeds (Pink Rajmah) and *Phaseolus mungo*, however, gradually decreased under identical conditions and duration of time. This indicates that while the proteins of diets A and C could be considered complete, from a nutritional point of view, the globulins of *Phaseolus vulgaris* (Pink Rajmah) and *Phaseolus mungo* cannot. This is further borne out by the amino acid analysis (Table V). However, significant difference in weight gain (15.0 ± 2.1 grams) of animals fed on diets A and C suggests that despite the efficiency of the two seed globulins with respect to essential amino acid content, their availability to the experimental animals from diet C is not adequate for proper growth.

A comparison of the amino acid composition of the two globulins of Pink Rajmah (diet B) and Red Rajmah (diet A), in view of the low capacity of diet B to promote growth in animals, points to the total absence of tryptophan and low methionine content. This is emphasized by the

Table V. Amino Acid Composition of Isolated Purified *Phaseolus* Seed Albumins[a] and Globulins

(Expressed as mg. amino acid/100 mg. dry proteins in terms of glycine)

	Albumins				Globulins			
Amino Acid	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Arginine	3.3	2.2	1.5	4.3	1.2	1.6	1.3	1.8
Histidine	1.2	2.8	1.0	1.1	1.5	1.0	1.4	0.8
Leucine-isoleucine	6.2	5.7	6.7	5.1	4.0	4.9	4.4	5.3
Lysine	3.4	2.6	3.2	4.2	5.2	2.3	3.1	2.1
Methionine	0.2	0.2	0.9	0.3	0.3	0.5	0.9	0.0
Phenylalanine	1.5	2.3	3.6	2.3	2.0	3.2	2.1	2.3
Threonine	3.2	3.6	4.2	2.3	2.6	3.3	4.2	3.1
Tryptophan[b]	0.0	0.0	1.1	1.0	0.0	0.7	0.6	1.0
Valine	2.2	3.7	1.1	3.2	2.0	2.8	3.4	3.2

[a] 1. *Phaseolus vulgaris* (Pink Rajmah).
2. *Phaseolus vulgaris* (Red Rajmah).
3. *Phaseolus vulgaris* (Bakla).
4. *Phaseolus mungo*.

[b] Mg. tryptophan/100 mg. dry proteins.

Table VI. Protein Efficiency Ratio and Net Protein Ratio of Dietary Proteins

Origin of Dietary Protein	Diet[a]	% N in Globulin	% Protein in Diet	Protein Intake/Rat in 5 Weeks, G.	Change in Weight/Rat in 5 Weeks, G.	Protein Efficiency Ratio	Net Protein Ratio
Red Rajmah	A	17.2	9.9	21.87 ± 0.67	+44.1 ± 3.8	2.1	2.7
Pink Rajmah	B	16.7	9.7	14.83 ± 0.63	− 6.1 ± 1.1	...	...
Bakla	C	17.8	10.1	21.30 ± 0.71	+29.6 ± 4.2	1.3	2.0
Pink Rajmah + tryptophan (0.2%, w./w.)	D	16.9	10.3	20.30 ± 0.91	+29.0 ± 2.1	1.4	2.1
Phaseolus mungo	E	18.1	10.1	13.72 ± 0.52	− 7.1 ± 1.8	...	...
Casein	F	17.8	9.9	21.10 ± 0.89	+48.1 ± 3.0	2.3	3.0
Nonprotein diet	G	...	...	...	−13.8 ± 1.3	...	...

[a] Nitrogen-free diet (G) consisted of soluble starch (analytical reagent grade) 80 parts; sucrose 10 parts; ground nut oil 6 parts; salt mixture 2 parts; shark liver oil 5 drops per rat (twice a week). In experimental diet, starch was replaced by dietary protein at 10% level.
Salt mixture prepared by mixing various components in the ratio (grams): $CaCO_3$, 543.0; $MgCO_3$, 25.0; $MgSO_4$, 16.0; K_2SO_4, 67.0; KH_2PO 112.6; $FePO_4$, $4H_2O$, 211.5; KI, 20.5; $MnSO_4.H_2O$, 0.08; NaF, 0.03; $Al_2(SO_4)_3.K_2SO_4.24H_2O$, 1.00; $CuSO_4.5H_2O$, 0.15.
Vitamin mixture supplied the following vitamins per 100 g. of diet: vitamin A, 400 I.U.; vitamin D, 44 I.U.; inositol, 2.2 mg.; choline chloride, 75 mg.; niacin, 2.0 mg.; riboflavin, 0.4 mg.; pyridoxine-HCl, 0.2 mg.; thiamine-HCl, 0.4 mg.; calcium pantothenate, 1.2 mg.; folic acid 40 mg.; vitamin B_{12} 600 μg.

Table VII. Nitrogen Balance Studies in Rats Fed on Dietary Proteins

Diets[a]	Nitrogen Intake/Rat, G.	Urinary Nitrogen Excreted/Rat G.	Fecal Nitrogen Excreted/Rat, G.	Total Nitrogen Excreted/Rat, G.	Net Nitrogen Retained/ Rat, G.	Metabolic Nitrogen, G.	Endogenous Nitrogen, G.	Biological Value
A	0.27 ± 0.02	0.096 ± 0.006	0.016 ± 0.002	0.113 ± 0.007	0.157	0.006 ± 0.001	0.009 ± 0.002	67
C	0.23 ± 0.03	0.109 ± 0.002	0.014 ± 0.003	0.123 ± 0.005	0.107	0.006 ± 0.001	0.009 ± 0.002	55
D	0.20 ± 0.01	0.118 ± 0.003	0.021 ± 0.002	0.139 ± 0.003	0.061	0.006 ± 0.001	0.009 ± 0.002	44

[a] Origin of dietary proteins in diet same as in Table VI.

biological assay experiments on laboratory animals. Supplementing diet B with DL-tryptophan (0.2% w./w.) enhanced growth (Table VI). However, the growth in the experimental animals even after supplementing with tryptophan was not as vigorous as in the case of Red Rajmah and casein diets, suggesting that the second limiting factor—the low concentration of methionine—was responsible for this and supplementation of adequate quantities could promote better growth.

The obvious effect of appropriate composition of essential amino acid content in experimental diets, both qualitative and quantitative, on the appetite of experimental animals suggests a correlation between composition and growth. Moreover, although the nutritional efficiency of a protein depends almost entirely upon its amino acid composition, certain other factors cannot be ignored. The physiological state of the experimental animals, for example, as well as the consequent uncontrollable changes in the rate of availability of amino acids to them are factors which vary by appreciable margins and significantly affect the nutritional efficiency of proteins.

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