PROTEIN SUPPLEMENTATION

Supplementary Value of INCAP Vegetable Mixture 9 for the Diets of Average School Children in Rural Guatemala

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Biological trials with rats were done to learn whether INCAP Vegetable Mixture 9, skim milk, and a mixture of equal weights of both could be satisfactory supplements in the diet of the average rural child in two towns in Guatemala. Both foods and the mixture of the two increased weight gain of the rats and the feed and protein efficiency of the rural diets. The supplementary effects were more marked for skim milk, the mixture of the two, and Vegetable Mixture 9 in this order. The poorer the quality of the average rural diet, the greater the effect of the supplement. The effect of the supplements on blood, bone, and liver composition was also studied with the best of the average rural diets. The supplements were not very different in affecting organ composition and all resulted in changes from the basal diet, more marked for the skim milk, the mixture of skim milk plus Mixture 9, and Mixture 9, in that order. The lower supplementary values of Mixture 9 as compared to skim milk were ascribed to the relative deficiency of lysine, the lower digestibility of Mixture 9, and the deficiency of lysine in the diets. None of the supplements reduced the intake of the average rural diet.

The results of dietary surveys in the Central American area have indicated low intake of good quality protein in the rural populations (*11*). Of particular importance is the quality of the protein consumed by the child population, the group most susceptible to dietary deficiencies. The diets of children in Guatemala are deficient in both quality and quantity (*13*). The protein consumed is derived mainly from corn and beans, with small and sporadic intakes of animal protein (*13*). INCAP Vegetable Mixtur9e [29% corn flour, 29% sorghum, 38% cottonseed flour, 3% torula yeast, and 1% $CaCO_3$; to 100 grams of the mixture 4500 I.U. of vitamin A (dry vitamin A acetate, 500,000 U.S.P. units per gram, Hoffmann-La Roche, Inc., Nutley, N. J.) are added] was developed to supplement the diets of the rural population (*8*). Biological tests with chicks (*5*), rats (*7, 9*), dogs and swine (*6*), and children (*20*) showed that Vegetable Mixture 9 was of high nutritive value, comparable to milk, particularly at high levels of protein intake. It was, therefore, of interest to study the supplementary value of its protein to the protein of the average child's diet in two towns in Guatemala.

Materials and Methods

Dietary surveys gave the amounts of food consumed per child per day in two rural towns in Guatemala: Santa María Cauqué and Santa Catarina Barahona. The selection of foods, the quantities consumed, and the proximate analysis of the air-dried diets are shown in Table I.

Food composites were made by homogenizing all foods in a Waring Blendor and dehydrating by hot air at 75° C. in a tray dryer. The dried samples were then ground to about 40-mesh and subsamples were obtained for chemical analysis and determination of amino acid content.

The biological tests in rats consisted in feeding the dried diets *ad libitum* and giving a daily supplement of 1, 2, 3, and 4 grams of Vegetable Mixture 9, skim milk, and a 50/50 mixture by weight of both. When the combination of skim milk and Vegetable Mixture 9 proteins was studied, the composition of the basal diet was: 4.0% mineral mixture (*15*), 5.0% cottonseed oil, 1.0% cod liver oil, 90% cornstarch, and 5 ml. of a vitamin solution (*19*). Skim milk and Vegetable Mixture 9 replaced part of the cornstarch to give the protein levels shown in Table II.

Table I. Mean Daily Food Consumption of Guatemalan Indian Preschool Children (Grams per Head) in Terms of Edible Portion

Food	*Santa Catarina Barahona*	*Santa María Cauqué*
Liquid milk	47	5
Eggs	5	4
Meat	9	14
Legumes	10	20
Fresh vegetables	41	33
Fruits	31	17
Tubers	3	4
Cereals[a]	119	178
Sugars	23	34
Lard	1	1
Coffee	5	3
Proximate Analysis		
Moisture, g. %	4.8	7.6
Ether extract, g. %	4.0	4.8
Crude fiber, g. %	2.5	2.7
Protein (N × 6.25), g. %	11.7	11.6
Ash, g. %	3.0	3.0

[a] Calculated on dry-weight basis.

Weanling rats of the Wistar strain from the INCAP colony were used. In each experiment the animals were distributed by weight and sex, three males and three females per group, having the same initial weight. They were placed in individual wire cages with raised screen bottoms. Weight gains and food intakes were measured every 7 days for the duration of the 28-day experimental period. One experiment was carried out for 8 weeks. Water was available at all times.

At the end of the 8-week experiment, using the diet of Santa Catarina Barahona, the animals were decapitated and blood and certain organs were obtained for chemical analysis. Nitrogen determinations were carried out by the Kjeldahl method, fat by ethyl ether extraction, and ash in an oven at 600° C. Calcium content of the ash was determined by the AOAC method (*3*), and phosphorus was determined by the method of Lowry and López (*17*). The bones were treated and analyzed as described by Braham *et al.* (*4*). The serum was analyzed for total protein by the method of Lowry and Hunter (*16*) and albumin by the method of Lowry *et al.* (*18*). Serum urea was determined by the method of Gentzkow and Mosen (*14*) and cholesterol by the method of Abell *et al.* (*1*). Hemoglobin was determined by Cannan method (*10*).

Table II. Combination of Vegetable Mixture 9 and Skim Milk Proteins at Two Levels of Dietary Protein in Rats

Protein Distribution in Diet, %		Amount of Food in Diet, %		Low Protein				High Protein			
VM9	Skim milk	VM9	Skim milk	Protein in diet, %	Weight gain,[a] grams	FE	PER	Protein in diet,[b] %	Weight gain,[c] grams	FE	PER
100	0	37.0	0	11.49	61	5.6	1.57	19.55	152	3.0	1.71
80	20	29.0	6.1	11.97	80	4.4	1.90	18.93	163	2.8	1.88
60	40	22.0	12.1	11.16	103	3.8	2.35	18.91	162	2.7	1.95
40	60	15.0	18.2	11.74	112	3.5	2.45	19.63	160	2.6	1.99
20	80	8.0	24.2	11.50	112	3.4	2.56	18.94	155	2.5	2.08
0	100	0	30.3	11.95	110	3.2	2.64	18.19	147	2.6	2.10

[a] Average initial weight, 43 grams.
[b] Amounts of Vegetable Mixture 9 and skim milk twice amounts shown for first experiment.
[c] Average initial weight, 52 grams.

VM9 = Vegetable Mixture 9; FE = feed efficiency; PER = protein efficiency ratio.

Table III. Supplementary Value of INCAP Vegetable Mixture 9 and of Skim Milk, Alone and Combined, in Child's Average Diet

Supplement, Grams/Day	Vegetable Mixture 9				Skim Milk				50/50 VM9 + Skim Milk			
	Weight gain,[a] grams	Basal food intake	FE[b]	PER	Weight gain,[a] grams	Basal food intake	FE[b]	PER	Weight gain,[a] grams	Basal food intake	FE[b]	PER
					Santa María Cauqué							
None	14	206	14.7	0.58	...	...	...	...	...	...	...	...
1	38	226	6.7	1.12	53	262	5.4	1.39	46	226	5.5	1.35
2	51	221	5.4	1.25	68	257	4.6	1.52	63	231	4.5	1.50
3	70	235	4.5	1.40	82	266	4.2	1.55	82	247	4.0	1.60
4	82	245	4.3	1.40	88	250	4.0	1.52	104	255	3.5	1.75
					Santa Catarina Barahona							
None	62	334	5.4	1.61	...	...	...	...	...	...	...	...
1	72	307	4.6	1.68	87	312	3.9	2.00	86	310	3.9	1.99
2	81	291	4.3	1.67	113	324	3.3	2.16	109	319	3.4	2.11
3	97	310	4.0	1.67	138	350	3.1	2.21	129	327	3.2	2.15
4	105	285	3.7	1.67	157	355	2.9	2.22	132	305	3.1	2.03

[a] Average initial weight for all groups. Santa María Cauqué, 52 grams; Santa Catarina Barahona, 53 grams.
[b] Value in feed efficiency blocks. Total food consumed/weight gain.

Results

Table II shows the results of two experiments designed to determine the optimum combination of the proteins of Vegetable Mixture 9 and skim milk. At the low level of protein in the diet, the replacement of vegetable protein by skim milk improved feed utilization and protein efficiency ratio. Only small increments in protein efficiency were obtained over that of diets containing 60% of the protein from Vegetable Mixture 9 and 40% from skim milk. Average weight gain of the rats also reached maximum values when the two food proteins were combined in these proportions. At the higher level of dietary protein, maximum weight gains were observed when the protein of the diet consisted of 80% derived from Vegetable Mixture 9 and 20% from skim milk. Protein efficiency ratio (PER), however, improved as the amount of skim milk increased. Here also only small increments in PER were noted over that of diets containing 60% of the protein from the vegetable mixture and 40% from skim milk.

Table III presents the values obtained when Vegetable Mixture 9, skim milk, and a 50/50 mixture of the two proteins were used to supplement the average diet of school children of Santa María Cauqué and Santa Catarina Barahona.

Table IV. Growth of Rats Fed Average Santa Catarina Barahona Diet Alone or Supplemented with 5 Grams Daily of Vegetable Mixture 9, Skim Milk, or Both

	4-Week Data				8-Week Data			
Treatment	Control diet intake, grams	Weight gain,[a] grams	FE	PER	Control diet intake, grams	Weight gain, grams	FE	PER
Control	250	52	4.8	1.80	621	116	5.3	1.62
+ skim milk	318	137	2.9	2.35	762	228	4.1	1.68
+ 50/50 mixture skim milk + VM9	302	137	3.0	2.10	722	221	4.3	1.53
+ VM9	277	109	3.5	1.74	664	194	4.6	1.40

[a] Average initial weight, 44 grams.

The daily addition of the vegetable mixture to the diet of Santa María Cauqué significantly improved weight gain, feed efficiency, and protein efficiency ratio. Optimum PER was observed when the supplement was 3 grams daily. Supplementation with skim milk also significantly improved weight gain and feed and protein efficiency. In this case, however, 1 gram daily of skim milk produced a PER similar to that obtained with 3 grams daily of the vegetable protein. The mixture of the vegetable protein and skim milk also improved weight gain and feed and protein efficiency significantly. In this case, larger amounts of the supplement caused greater increases in PER and 1 to 2 grams daily had the same effect as 1 gram of skim milk or 3 grams daily of the vegetable protein. In all cases, the addition of the supplements increased the basal diet intake.

When the same supplements were used with the average diet of school children of Santa Catarina Barahona, INCAP Vegetable Mixture 9 caused only small increases in PER, although weight gains were significantly increased by supplementing with 2, 3, and 4 grams daily. Supplementation with skim milk significantly improved weight gain and feed and protein efficiency ratio. The addition of only 1 gram of skim milk daily improved PER more than 4 grams of the vegetable mixture. Daily supplementation with 1 gram of the skim milk–vegetable protein mixture improved weight gain and feed and protein efficiency ratio as much as 1 gram of skim milk. As with the previous experiment, the supplements increased the consumption of the basal diet.

Table IV shows the results of another experiment in which 5 grams daily of the three protein supplements were added to the diet from Santa Catarina Barahona. Weight gain and feed and protein efficiency values are shown for 4- and 8-week experimental periods. Both skim milk alone and the combination of skim milk and the vegetable protein improved PER. All three supplements increased weight gain of the rats. As expected, larger differences in PER were noted at 4 than at 8 weeks.

Table V shows the values of some blood constituents of the rats included in the foregoing experiment. All supplements increased total serum protein, globulin, and serum urea nitrogen. Albumin content was increased by the vegetable mixture alone and combined with skim milk. Hemoglobin concentration increased with either supplement, while cholesterol content decreased with the addition of any of the supplements. The results in Table VI show the average weights of liver, spleen, kidney, heart, and carcass expressed as percentage of body weight of the animals fed the different supplements. Kidney, heart, and spleen weights were significantly different between treatments.

Table VII shows the composition of the long bone of the rats. The moisture content of the bones from rats fed the skim milk supplement was significantly different from the other values. The differences in fat, ash, and protein were also significant between treatments. The amount of calcium and phosphorus in the bones was increased by supplementation with skim milk or the mixture of skim milk and Vegetable Mixture 9, the differences being statistically significant. Also shown in Table VII is the moisture, fat, and protein content of the livers of the animals fed the diet and the diet plus the supplements. The moisture content of the liver of the animals fed Vegetable Mixture 9 as the supplement was lower and significantly different from the other values. The fat content was higher in the control, but the differences were not significant between the control and any of the supplements or between supplements. Protein concentration in the liver was statistically different between treatments. Higher values were observed when the supplement was skim milk.

Discussion

Previous studies have demonstrated that the protein value of INCAP Vegetable Mixture 9, when fed at low protein levels, is not so good as that of milk because of a lower protein digestibility (*20*) and a relative deficiency of lysine (*7*). At high level of dietary protein, the nutritive value of the mixture is comparable to that of milk protein. These findings explain the results obtained when the proteins of skim milk and of Vegetable Mixture 9 were combined. As milk protein replaced Vegetable Mixture 9 protein, more lysine was available to the animals, resulting in improvements in gain in weight and PER. The lysine deficiency in the vegetable protein is more evident at the lower protein level, as seen from the results obtained at the two levels of protein in the diet. In any event, the best combinations of the two proteins lie between 60% of the protein from Vegetable Mixture 9 and 40% from milk and 20% from the vegetable protein and 80% from milk at the lower level of protein fed.

Studies (*12*) on the chemical composition, amino acid content, and protein value of the average rural child's diet have shown that both Santa Catarina Barahona and Santa María Cauqué diets contain around 11% protein on the dry weight basis and that lysine is the first limiting amino acid; the diet from Santa María Cauqué is the more deficient of the two. The diets contain 3.8 and 3.3 grams of protein from animal origin per day, respectively. The improvement in the weight gain of the rats and in the PER upon supplementation, particularly with the skim milk and skim milk–vegetable protein supplement, is due to better protein quality as well as a higher intake of protein from the supplement. This can be observed from the PER values, which increase to a maximum value and remain constant or decrease slightly with higher amounts of the supplement, an effect similar to the decrease in PER with increasing levels of dietary protein (*2*). That the protein quality of the Santa Catarina Barahona diet is better than that of the Santa María Cauqué diet is shown by the basal PER values as well as the improvement in PER upon supplementation, which is large for all supplements in the case of the diet with the lower basal PER. It is evident that the nutritive value of the protein from the vegetable mixture is about $^2/_3$ that of the protein derived from skim milk.

The results of this study also point to an important factor related to the

Table V. Blood Serum Analysis of 8-Week-Old Rats Fed Average Santa Catarina Barahona Diet, Alone or Supplemented with Vegetable Mixture 9, Skim Milk, or Both

Group No.	Treatment	Protein, %	Albumin, %	Globulin, %	A/G Ratio	Urea N, Mg. %	Hemoglobin %	Cholesterol, Mg. %
1	Control	5.43	3.31	2.18	1.55	16.7	13.4	101.2
2	Skim milk	6.43	3.14	3.29	0.96	17.5	14.6	82.4
3	Skim milk + VM9	6.57	3.38	3.20	1.06	19.6	14.6	88.9
4	VM9	6.20	3.42	2.78	1.24	20.2	14.6	87.7

Table VI. Organ and Carcass Weight of 8-Week-Old Rats Fed Average Santa Catarina Barahona Diet Alone and Supplemented with Vegetable Mixture 9, Skim Milk, or Both

Group No.	Treatment	% of Body Weight				
		Liver	Kidney	Heart	Spleen	Carcass
1	Control	5.60	1.68[a]	0.85[b]	1.08[b]	82.2
2	Milk	4.37	1.17	0.64	0.69	81.9
3	Milk + VM9	4.19	1.11	0.63	0.57	82.4
4	VM9	4.76	1.21	0.48	0.63	82.8

[a] Difference between groups significant at 0.05 level.
[b] Difference between groups significant at 0.01 level.

Table VII. Chemical Composition of Long Bones and Liver of 8-Week-Old Rats Fed Average Santa Catarina Barahona Diet Alone or Supplemented with Vegetable Mixture 9, Skim Milk, or Both

Group No.	Treatment	Bone						Liver, % Fresh Tissue		
		% Fresh Tissue				% of Ash				
		Moisture	Ether extract	Ash	Protein	Calcium	Phosphorus	Moisture	Ether extract	Protein
1	Control	41.4	3.7	31.7	22.7	29.6	16.2	67.90	5.42	33.46
2	Milk	34.4	1.6	30.4	19.4	44.7	22.6	67.57	3.79	42.53
3	Milk + VM9	36.1	1.7	30.6	19.3	42.0	20.4	66.01	3.84	41.63
4	VM9	41.8	4.9	30.2	20.4	31.3	13.5	65.17	3.61	40.09

development of vegetable protein mixtures for supplementing human diets. These mixtures usually consist of combinations of a high protein source and a cereal grain, as is the case with Vegetable Mixture 9. They are to be used to supplement rural diets rich in protein-poor cereal grains. Therefore, the vegetable mixture should consist of the optimum protein combination of the cereal protein and the protein-rich component to give the highest nutritive value that can be obtained. Otherwise, the effect on the rural cereal diet will not be as efficient as expected.

Although the supplementary effect of Vegetable Mixture 9 was not as high as expected, it cannot be concluded that it would not be of value when diets, such as the ones used in this study, are consumed by children. The rat is a faster growing organism than the human and consequently has a higher lysine requirement.

All supplements (except in the Santa Catarina Barahona diet) did not reduce food intake, a further indication of the improvement in quality of the supplemented diets. A disadvantage of vegetable protein mixtures is their bulk and low digestibility.

The supplements, besides improving weights gain and PER, also improved some of the constituents of the tissues analyzed and increased the weight of some of the organs studied. The bone composition data, however, suggest that calcium is not as effectively utilized in these mixtures as the calcium from skim milk; this could be due, to some extent, to the phytin content of the vegetable products, and could explain, to a certain degree, the lower PER values obtained with the mixture as compared to those obtained with skim milk.

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Received for review February 19, 1965. Accepted August 2, 1965. Investigation supported by grants RF-NRC-1 from the National Research Council (U. S.) and A-981 from the National Institutes of Health. INCAP Publication I-309.

NEW ALFALFA COMPOUND IDENTIFIED

Isolation of 4′-O-Methylcoumestrol from Alfalfa

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The coumestan 4′-O-methylcoumestrol was tentatively identified by thin-layer chromatography (TLC) and comparison of its benzyloxybenzofuran derivative with authentic 2-(2′-methoxy-4′-benzyloxyphenyl)-6-methoxybenzofuran. Comparison of the isolated product with authentic 4′-O-methylcoumestrol confirmed the assigned structure.

In a recent paper (*2*), the isolation of 13 phenolic compounds from alfalfa was reported. Two of these (compounds I and II) were obtained as an inseparable mixture. That the two compounds were closely related coumestans was suggested by the similar ultraviolet spectrum of the mixture to that of coumestrol (*1*). Chromatography established that neither compound was coumestrol (*2*). Formation of two new compounds upon acetylation or methylation of the mixture suggested the presence of hydroxyl groups on both compounds. Compound I was subsequently identified through degradative and proton magnetic resonance (PMR) studies as 7-hydroxy-11,12-methylenedioxycoumestan and assigned the trivial name medicagol (*4*). TLC comparison of the mixture of medicagol and compound II with 7- and 4′-*O*-methylcoumestrol suggested that compound II was 4′-*O*-methylcoumestrol (Table I).

The definite location of the hydroxyl group on compound II was established by the preparation of the benzyloxybenzofuran derivatives of the mixture of medicagol and compound II. These were prepared by the systematic degradation of their benzyloxy derivatives through methylative ring opening, hydrolysis, and decarboxylation to their benzyloxybenzofuran derivatives. The benzofurans were then separated by fractional crystallization. Elemental analyses of the benzyloxybenzofuran derivative of compound II substantiated that the compound from which it was derived was a monomethoxy derivative of coumestrol. Comparison with authentic 2 - (2′ - methoxy - 4′ - benzyloxyphenyl) - 6 - methoxybenzofuran confirmed its identity.

Natural 4′-*O*-methylcoumestrol was obtained by treatment of the mixture of compound II and medicagol with 86% sulfuric acid. Under the conditions employed, medicagol was completely converted to 7,11,12-trihydroxycoumestan, while 4′-*O*-methylcoumestrol re-