samples were nearly equal to the values for milk and lactalbumin, slightly lower than egg white (except for wheat samples 539, 549, and 552, and corn 573a, which were about equal to egg white), and much lower than whole egg. On the other hand, all LPC samples except IR4 had estimated biological values higher than those of soybean, yeast, wheat flour, gluten, zein, and gelatin.

There was relatively little difference among the leaf proteins from nine species of the plants in their amino acid composition, hydrolysis by digestive enzymes, and estimated biological value. This suggests that leaf proteins from a large number of plant species growing in different localities would have uniformly high biological value. The plant species appear to differ mainly in the total yield of LPC which can be obtained from the leaves, not in the digestibility or amino acid composition of leaf proteins.

The authors have concluded that leaf proteins may be an excellent source of protein for animal or human consumption. Furthermore, leaf protein concentrates could be prepared in nearly every part of the world where green plants grow.

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LOW-COST PROTEIN SOURCES

Protein-Rich Mixture Based on Vegetable Foods Available in Middle Eastern Countries

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A protein-rich mixture of vegetable foods available in Middle Eastern countries consists of 47% autoclaved chick peas, 35% defatted sesame flour, and 18% heat-processed low-fat soybean flour, and contains 37.8% protein. Its biological value is 74 and its protein efficiency ratio (assessed on young rats in 28 days' assays) is 2.90. Comparison with the FAO egg protein pattern suggests that the order of limiting amino acids is methionine, followed by tryptophan. The mixture is a good source of B vitamins, calcium, and iron.

In various regions of the world where protein deficiency in infants constitutes a major problem of public health and nutrition, efforts are being made to introduce into the diet protein from locally available sources, particularly of vegetable origin. The preparation must be cheap and the population concerned be well acquainted with its constituents. Such protein-rich foods, which may be made of various components, have been used in India (*11, 19*) and Central America (*6, 18*).

Protein deficiency in infancy is frequently encountered in Middle Eastern countries (*9, 14, 17, 22*). Therefore, a protein-rich mixture of locally available cheap foods was needed, particularly for infant feeding. Since high nutritive value of the protein was thought to be of great importance, the concentrations of essential amino acids per gram of nitrogen should not be much less than those suggested by the Food and Agriculture Organization (*8*). Various mixtures containing different proportions of wheat flour, parboiled wheat (local name, burgul), soybean flour (*Glycine max.* L.), sesame flour (*Sesamum indicum* L.), chick peas (*Cicer arietinum* L.), and sunflower seed meal (*Helianthus annus* L.) were studied, and the amounts of the amino acids limiting the nutritive value of most proteins—i.e., methionine, lysine, and tryptophan—were determined. After several trials a mixture of chick peas, defatted sesame flour, and toasted, defatted soybean flour seemed to deserve further study. Since the mixture is intended to be used by small children, the chick peas were autoclaved in order to avoid possible intestinal disturbances.

Materials and Methods

Protein Sources. Chick peas obtained on the open market were autoclaved for 30 minutes at 15 pounds' pressure, dried in the sun, and finely milled. Sesame flour was prepared from decorticated, crushed, and roasted seeds (local name, tehinah). The seeds were extracted with ether and excess solvent was removed by exposure to the sun without additional heating. Fat-extracted toasted soybean flour was obtained from a local manufacturer. For comparison of nutritional values, lactalbumin and wheat gluten (Nutritional Biochemicals Corp., Cleveland, Ohio) were used.

Analytical Procedures. Nitrogen was determined by the Kjeldahl method. One gram of nitrogen was assumed to be equivalent to 6.25 grams of protein.

For estimation of amino acids (except tryptophan) the samples were hydrolyzed for 24 hours with 8N H_2SO_4. Tryptophan was released enzymatically using pepsin and trypsin as described by Barton-Wright (*5*, p. 152). The liberated amino acids were determined microbiologically (*5*).

For determination of B vitamins the samples were digested as described by the Association of Vitamin Chemists (*3*). Thiamine was determined by the thiochrome method (*3*), riboflavin fluorometrically (*3*), and the other vitamins microbiologically—i.e., niacin and pantothenic acid with *Lactobacillus arabinosus* 17-5 and folic acid with *Streptococcus faecalis* (*3*).

For determination of calcium the ash was dissolved in 1N HCl and the procedure of Baron and Bell (*4*) adopted. Iron was determined by the American Association of Cereal Chemists' method (*1*).

Biological Methods. Biological tests involved both rats and chicks. For rat assays diets containing 9% of protein were prepared. The protein source tested was diluted with a protein-free diet which consisted of cornstarch (if not otherwise stated) 91 parts, vegetable oil 5 parts, and salt (U.S.P. XIV) 4 parts, and was supplemented with thiamine 0.2, riboflavin 0.3, pyridoxine 0.2, calcium pantothenate 1.6, and choline chloride 100 mg. per 100 grams of ration. Each rat received 100 I.U. of vitamin A twice weekly. Animals were kept in individual cages with raised screen bottoms.

Protein efficiency ratio (PER) was assayed on male weanling rats of a local strain weighing approximately 40 grams. The experiments lasted 28 days. The biological value (BV) and true digestibility coefficient were determined on male rats weighing 200 to 250 grams, using the nitrogen balance method. In the first balance, endogenous nitrogen was determined experimentally by giving the animals the protein-free diet. Other diets included those listed in Figure 1 and Table I. The animals were given a 4-day adaptation period which preceded the balance study of 5 to 6 days on each diet. In repletion assays male rats of similar weight were fed a protein-free diet for 2 weeks, followed by the test diet for 10 days. The nutritive value of the protein was expressed as the ratio of weight gain to protein consumed.

The chick experiments were performed on three groups of five 5-day-old White Leghorn chicks for each protein. The diet and method described by Ascarelli and Gestetner (*2*) were adopted.

Table I. Biological Value and True Digestibility Coefficient of Protein Mixture, Incap Vegetable Mixture 9, and Indian Multipurpose Food

	Protein Mixture		*Incap Vegetable Mixture 9*		*Indian Multipurpose Food*
Carbohydrate added to test diet	Cornstarch	Sucrose	Glucose	Cornstarch	Cornstarch
No. of animals	8	17	19	9	10
Biological value, %[a]	72 ± 2.6[b]	74 ± 2.6	76 ± 3.6	74 ± 3.3	71 ± 3.0
True digestibility coefficient, %[c]	82 ± 2.6	80 ± 1.3	84 ± 1.0	89 ± 0.9	90 ± 0.5

[a] Proportion of absorbed nitrogen retained in body for maintenance.
[b] All results expressed as averages ±SE of mean.
[c] Proportion of food nitrogen absorbed.

Protein source	1	2	3	4	5	6	7	8	9	10	11
	% of total protein										
Chick peas, raw	100	-	-	-	25	50	25	33	-	-	-
Chick peas, autoclaved	-	100	-	-	-	-	-	-	25	-	-
Sesame flour	-	-	100	-	50	25	25	33	50	-	-
Soybean flour	-	-	-	100	25	25	50	34	25	-	-
Lactalbumin	-	-	-	-	-	-	-	-	-	100	-
Wheat gluten	-	-	-	-	-	-	-	-	-	-	100
NUMBER OF ANIMALS:	12	8	8	12	22	10	10	9	8	12	12

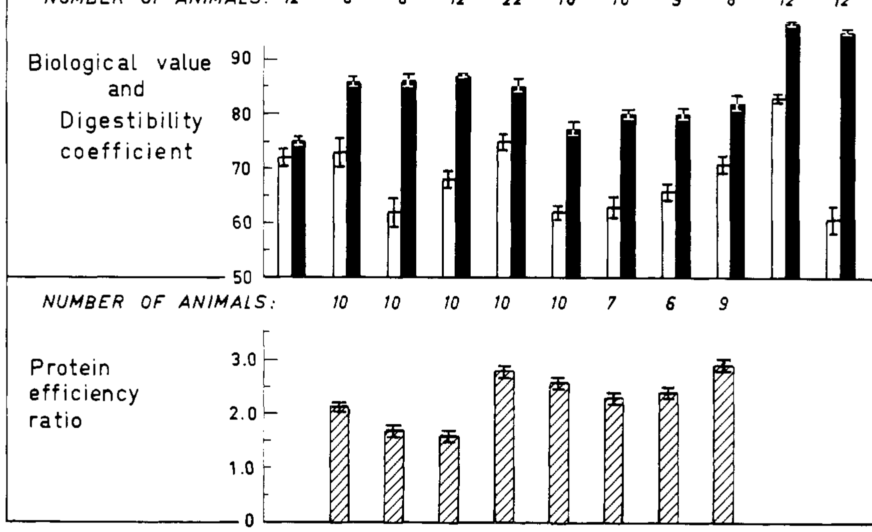

Figure 1. Biological value, digestibility coefficient, and protein efficiency ratio in rats of chick peas, sesame flour, and soybean flour and their combinations

White columns represent biological values, black columns digestibility coefficients, and shaded columns protein efficiency ratios. Twice the standard error of each mean is represented by a vertical line through the mean

Results

Nutritional Value of Protein. In the first series of experiments the BV, true digestibility coefficient, and PER of proteins of raw and autoclaved chick peas, sesame flour, and soybean flour and their combinations were determined. BV's and digestibility coefficients were compared with the corresponding values obtained with lactalbumin and wheat gluten—i.e., proteins of high and low nutritive value (Figure 1).

Mixtures containing chick peas as well as sesame flour and soybean flour have a higher PER than their components alone. The highest BV and PER were found with a mixture in which 50% of the protein was derived from sesame flour and 25% each from chick peas and soybean flour (sources 5 and 9). Both digestibility and BV were slightly higher with source 5 containing the raw chick peas than with source 9 containing the autoclaved peas. A higher PER, however, was noted with source 9. The nutritional value of lactalbumin was markedly higher and that of wheat gluten considerably lower (sources 10 and 11). Autoclaving chick peas improves their digestibility (sources 1 and

2) but has no marked effect on the nutritional value of protein mixtures in which this protein provides 25% of total protein (sources 5 and 9). Mixture 9, "protein mixture," was, therefore, chosen for further study. It consists of 47% of chick peas (20% protein), 35% sesame flour (55% protein), and 18% soybean flour (51% protein). It contains 37.8% protein.

The BV of the protein mixture was assessed with an experimental diet in which cornstarch constituted the source of carbohydrates. In another experiment this polysaccharide was replaced by a disaccharide or a monosaccharide—i.e., sucrose or glucose. The results are presented in Table I.

The different carbohydrates incorporated in the test diet had no significant effect on the biological value of the protein mixture. With glucose as the main source of carbohydrate digestibility was slightly higher than with sucrose.

Next, BV of the protein mixture was compared with that of two other vegetable protein preparations—Incap vegetable mixture 9 and Indian multipurpose food. The test diets included cornstarch as a source of carbohydrates. They were prepared as previously described, and contained 9% protein (Table I).

The BV of the protein mixture is similar to those of the other preparations, and its true digestibility is slightly inferior. The low digestibility coefficient of the protein mixture is unexplained, since the digestibility coefficients of its components—i.e., chick peas, sesame flour, and soybean flour—were higher (86, 87, and 87).

The ability of the protein mixture to promote weight increase of protein-depleted animals was tested in 10 rats. During a 10-day observation period they gained 2.40 (standard error, 0.12) grams per gram of protein consumed. Corresponding figures obtained in similar assays for lactalbumin and wheat gluten were 3.60 (0.32) and 1.30 (0.15).

It has been suggested (*20*) that new protein preparations should biologically be assessed on at least two different species of animals. Therefore, the nutritive value of the protein mixture was assayed on chicks using the method developed by Ascarelli and Gestetner (*2*). The results (Table II) are compared with corresponding values obtained by these authors for fish meal, cottonseed meal, and heat-processed soybean meal (*2*). The nutritive value of the protein mixture as assayed by this method appears superior to proteins of fish, cottonseed, and soybean meals.

Amino Acids, Vitamins, and Minerals. Essential amino acids were estimated in four samples of the protein mixture; each assay was carried out three times (Table III). The amounts of amino acids are compared with the egg pattern of FAO (*8*) and Incap vegetable mixture 9 as reported by Bressani *et al.* (*6*).

Table II. Nutritive Value of Protein Mixture as Assayed on Chicks

	Net Protein Utilization[a]	Net Protein Ratio[a]	Protein Retention Efficiency[a]
Protein mixture	68.7	4.0	68.5
Fish meal (6 samples)[a]	41.0–56.9	2.5–3.5	42.7–59.2
Cottonseed meal[a]	49.2	2.8	47.5
Soybean meal[a]	63.2	3.7	63.6

[a] Ascarelli and Gestetner (*2*).

Table III. Amino Acid Content of Protein Mixture, FAO Egg Pattern, and Incap Vegetable Mixture 9

Amino Acid	Protein Mixture,[a] Mg./Gram N Range	Protein Mixture,[a] Mg./Gram N Mean	FAO Egg Pattern,[b] Mg./Gram N	Score	Incap Vegetable Mixture 9,[c] Mg./Gram N
Lysine	347–371	360	396	91	343
Sulfur-containing					
Methionine	111–150	124	196	63	111
Cystine	55–121	77			
Total	166–271	201	342	59	192
Tryptophan	54– 94	71	106	67	59
Isoleucine	333–413	387	428	90	254
Leucine	330–481	423	565	75	473
Phenylalanine	352–393	366	368	99	Not stated
Valine	417–518	468	460	102	259
Threonine	205–257	237	310	75	198
Histidine	210–249	226			227

[a] Four samples assayed. [b] Food and Agriculture Organization (*8*). [c] Bressani *et al.* (*6*).

The protein mixture is composed of less sulfur-containing amino acids, tryptophan, leucine, and threonine than the egg pattern but similar amounts of the other essential amino acids. It compares favorably with Incap vegetable mixture 9, which contains slightly less lysine, sulfur-containing amino acids, and tryptophan.

Further studies included the estimation of five B vitamins, calcium, and iron. Three samples each of chick peas, sesame flour, and soybean flour, and four samples of the protein mixture were examined in duplicate or triplicate (Table IV). The protein mixture contains considerably more thiamine and riboflavin than chick peas, more niacin than chick peas and soybean flour, more pantothenic acid than sesame flour and chick peas, more folic acid than sesame flour, and more calcium than chick peas and sesame flour. No large differences were found with respect to iron.

Discussion

A protein-rich mixture of vegetable foods available in Middle Eastern countries was developed, which has a nutritive value higher than that of its components when assayed with growth tests performed on young rats. Its superiority was less evident when its proteins were evaluated in adult rats and with the nitrogen balance method.

Legume proteins are known to be deficient in sulfur-containing amino acids (*16*). Lysine is the limiting essential amino acid in sesame protein, which is a good source of sulfur-containing amino acids (*10*). Thus, the protein mixture includes more sulfur-containing amino acids than chick peas and soybean flour, and more lysine than sesame protein. Therefore, Krishnamurthy *et al.* (*12*) were able to improve the nutritive value of the proteins of Indian legume blends (Bengal gram, peanuts, soybeans) by supplementation with sesame flour. The nutritive value of the proteins of the present mixture was similar to that of other vegetable protein preparations used in India and Central America.

The nutritive value of the proteins of many legumes is increased by proper heat treatment (*13*). In experiments conducted in this laboratory chick peas were autoclaved for 10, 20, and 30 minutes. Protein digestibility rose from 75 in raw chick peas to 86 after 30 minutes' autoclaving; the biological value was not much increased. Hence, a 30-minute autoclaving was adopted.

The type of carbohydrate incorporated into the experimental diet had no marked effect on the biological value of the protein mixture. Digestibility was only slightly improved when glucose replaced sucrose as the main carbohydrate. Using sucrose and dextrin as sources of carbohydrates Wiener, Yoshida, and Harper (*21*) noted that the type of carbohydrate affected neither protein digestibility nor nitrogen retention. Digestibility of wheat gluten was not affected by substitution of sucrose or glucose for cornstarch in the test diet (*7*). It seems, therefore, improbable that the kind of carbohydrate in human diets to which the protein mixture may be added will markedly affect its nutritive value.

Table IV. B Vitamins, Calcium, and Iron in Sesame Flour, Chick Peas, Soybean Flour, and Protein Mixture

	Sesame Flour	Chick Peas	Soybean Flour	Protein Mixture
Thiamine, mg.	2.40–2.56[a] 2.53	0.42–0.56 0.46	1.10–1.50 1.26	1.08–1.45 1.25
Riboflavin, mg.	0.23–0.30 0.27	0.15–0.18 0.16	0.40–0.44 0.42	0.23–0.24 0.24
Niacin, mg.	10.6–13.6 12.6	1.16–2.85 2.28	2.03–2.91 2.36	4.93–5.56 5.13
Pantothenic acid, mg.	2.68–2.80 2.76	2.10–3.06 2.53	4.70–5.06 4.88	3.90–4.83 4.33
Folic acid, µg.	28–30 29	79–100 87	79–86 82	40–72 54
Calcium, mg.	124–163 150	150–161 156	416–636 526	220–260 246
Iron, mg.	14.0–16.0 14.3	15.0	11.0–16.0 12.9	12.0–7.0 13.5

[a] Per 100 grams.

Finally, the mixture contained considerable quantities of B vitamins, calcium, and iron. In this respect also it appears to be superior to its components.

In preliminary trials the protein mixture was administered to infants in order to study acceptability and tolerance. Water and sugar were added to make it isocaloric and isonitrogenous to cow's milk. After being cooked for 10 minutes it was given to 20 infants who did not suffer from gastrointestinal disorders. The preparation was well accepted and tolerated (*15*).

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CLOVER CONSTITUENTS

Isolation of Phenolic Compounds from Ladino Clover

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Previous studies at this laboratory on the phenolics of ladino clover have resulted in the isolation or detection of five estrogenlike compounds, coumestrol (*1, 2*), genistein, daidzein, formononetin, and biochanin A (*3*). In addition to estrogenic activity, about 33 different physiological and biochemical activities have been reported for 30 of the naturally occurring flavonoids of plants (*5*). The present investigation was a search for new, potentially valuable phenolic compounds in ladino clover.

Extraction and Fractionation

Step 1. Commercially dehydrated, pelleted ladino clover meal was ground, and 227 kg. of the ground meal were mechanically stirred for 24 hours with 190 liters of Skellysolve B at a temperature of 50° to 60° C. The mixture was filtered and the meal was dried under a stream of air, then extracted by heating and stirring with three 190-liter portions of acetone. After each extraction, the mixture was filtered, and the acetone extracts were combined and concentrated to a final volume of 30 liters containing 5.5 kg. of solids. Figure 1 presents a schematic two-dimensional