Effect of Age of Sample and of Amino Acid Supplementation on the *Tetrahymena*-Relative Nutritive Value of Lentils, Green and Yellow Split Peas, and Their Processed Forms[1]

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ABSTRACT

In this study, the protein quality of lentils, green and yellow split peas, and their processed forms was evaluated using *Tetrahymena pyriformis* w. The samples, which had been stored in the laboratory for two years, were tested with no treatment, with fat extracted by ether, and with the addition of the amino acids methionine (M), cystine (C), M + C, and M + C + lysine (L). In this study, the effects of storage, defatting, and amino acid supplementation were evaluated. The amino acid supplements permitted testing of the hypothesis that the tannins of the lentil seed coat depress growth of the organism by binding the amino acids M, C, and L. Significant changes in T-RNV were associated with storage, fat extraction, and amino acid supplementation. Storage was associated with a decrease in T-RNV of all lentil products, and with all green pea products except the pea flour. Storage was associated with an increase in the T-RNV of all yellow pea products except the precooked powder. Defatting increased the T-RNV of green pea precooked powder and decreased the T-RNV of the yellow precooked powder and protein concentrate. Defatting had only a slight effect on other pea products. Addition of the amino acids improved the T-RNV of all products, with the effects ranked in order of decreasing severity, CM, M, CML, C, none. Because the addition of lysine had such a small effect, and because the change in T-RNV was not greater in the high-tannin lentil products, the growth depression of tannins is not due to a simple binding of M, C, or L, but tanning must inhibit digestion by binding enzyme, substrate or cofactor, have a direct toxic effect on the organism, or inhibit absorption of amino acids.

Cereal Chem. 61(4): 311-315

The *Tetrahymena* assay for protein nutritional quality (T-RNV) has some advantages over the currently used rat protein efficiency ratio (PER). The measurement of the relative nutritive value of proteins using *Tetrahymena* can be done in less than a week, whereas the PER takes 28 days. The T-RNV can be done with gram quantities of sample, which could make it valuable in screening for grains or legumes of higher nutritional protein quality. However, if the assay is to be of value, the response of the organism to natural constituents of foods and to alterations in both food samples and assay media must be documented. Although neither the rat nor *Tetrahymena* have amino acid requirements identical to those of humans, both have been used to estimate the ability of protein sources to meet human needs. It is beyond the scope of this research to correlate *Tetrahymena* or rat-based results with human balance studies or requirements. The major objective of our study is to gain baseline information that might help to standardize the assay, to delineate factors other than protein that could interfere with results, and to suggest that the *Tetrahymena* assay might be a suitable rapid, inexpensive replacement or substitute for the standard rat-based PER.

Tannins are known to inhibit digestibility of proteins (Chang and Fuller 1964, Glick and Joslyn 1970, Ford 1978, Mondragon and Gonzales 1978, Ronnenkamp 1977, Davis 1981). The effects of tannins might be a result of the ability of the tannins to cross-link proteins by reacting with lysine or with methionine and cystine (Sosulski 1979). Tannins also have the ability to bind metallic cofactors or enzymes. Dryden et al (1978) observed marked depression of growth of *Tetrahymena* in response to chlorogenic acid. McCurdy et al (1978) reported that the organism grew less than expected when lentils were the source of protein. Davis (1981) also reported that lentils supported a smaller growth of the organism than did green or yellow peas, and that there was an apparent relationship between the tannin content of lentils and the depressed growth. If the tannins in the lentil seed coat are responsible for the depressed growth, and if the depressed growth is due simply to the unavailability of lysine, methionine and/or cystine, then supplementation with those amino acids should have a greater effect on the growth of the organism on lentils than on green or yellow peas.

This article presents results of experiments designed to evaluate the effect on the T-RNV of lentil (*Lens culinaris*), and of green or yellow split peas (*Pisum sativum*) and their processed products of two to three years of storage; of removal of the ether-soluble fat; and of supplementation with cystine, methionine, cystine + methionine, and cystine + methionine + lysine.

MATERIALS AND METHODS

Lentils, green and yellow split peas, precooked pea and lentil powders, and sterilized pea flours were purchased from Dumas Seeds, Inc. A portion of the lentils and peas were reserved and the rest sent to the Prairie Regional Laboratory, Saskatoon, Saskatchewan, Canada, where they were pin-milled and air-classified to produce the protein and starch concentrates. The sterilized flours are produced by subjecting the peas to a short steam treatment designed to inactivate the enzymes.

The products were obtained in the spring of 1979, and initial analyses were made during 1979–1980. The samples were stored in a laboratory where temperatures were maintained at approximately 68°F during the winter and 80°F during the summer, and where the relative humidity was about 35%. Analyses for the present study were made during 1982. To determine whether observed differences were due to the age of a sample or to other experimental treatments, T-RNV analyses were performed on the samples before other treatment, as well as after defatting and with amino acid supplements. Portions (approximately 5 g) of the peas and pea products were defatted for 4 hr, using ethyl ether on a Goldfisch fat-extraction apparatus. The defatted samples were allowed to stand in the hood overnight so that the ether would dissipate. Then they were dried at 65° C in a vacuum oven for 4 hr. The nitrogen content of the defatted samples was determined by micro-Kjeldahl analysis.

The T-RNV assays were performed as previously reported (Davis 1981). For the analysis, the samples were weighed into plastic centrifuge tubes and then dispersed in water. The pH was adjusted and pancreatin added. The samples were predigested for about 2 hr. The hydrolysates were diluted to provide 0.3 mg of nitrogen per milliliter. For the amino acid supplementation studies, the supplements were made in aqueous solutions as follows: 5.33 μg of cystine, or 7.25 μg of methionine, or 5.33 μg of cystine + 7.25 μg

[1] Published with the approval of the director of the Idaho Agricultural Experiment Station as Research Paper No. 8357.
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of methionine, or 5.33 μg of cystine + 7.25 μg of methionine + 13.05 μg of lysine per milliliter. To maintain a final volume of 5 ml in the flasks for the assays, the 2.5 ml of growth medium added to the samples without supplementation was replaced with 1.25 ml of double-strength medium and 1 ml of supplemental amino acids in water plus 0.25 ml of water. The samples were inoculated with a three-day-old subculture of *Tetrahymena* and incubated at 26 ± 1°C for three days. Growth was stopped, and the organisms preserved by the addition of 1 ml of isotonic formalin to 1 ml of sample. The organisms were enumerated by direct microscopic count, using a hemacytometer with a modified Neubauer ruling.

Analysis of variance (ANOVA) within type of legume was done by using a factorial design. The processed form was considered to be the major factor, and age of sample and defatting were minor factors. In the factorial design to evaluate the effects of amino acid supplementation, processed form and variety were major factors, and amino acid supplements were minor factors. Since lentil flour was unavailable, the ANOVA was divided into two models. The first model incorporated three legume types, four processed forms (omitting flour), and five supplements. The second model incorporated two legume types (omitting lentils), five processed forms, and five supplements.

TABLE I
ANOVA of the Effects of Age of Sample and of Defatting on the T-RNV of Green and Yellow Peas and Pea Products, and of Age of Sample on Lentils and Lentil Products

Source	Degree of Freedom	Mean Squares	F[a]
Green peas			
Age-defatting	2	31.7	2.51**
Processed form	4	391.4	31.10**
Interaction	8	57.2	4.55**
Replication	2	18.7	1.49
Error	28	12.6	...
Total	44	...	...
Yellow peas			
Age-defatting	2	776.1	87.4**
Processed form	4	181.8	40.9**
Interaction	8	146.6	16.5**
Replication	2	7.4	0.8
Error	28	8.9	...
Total	44	...	...
Lentils			
Age	1	569.4	44.8**
Processed form	3	536.2	42.2**
Interaction	3	26.1	2.0
Replication	2	7.3	0.6
Error	14	12.3	...
Total	23	...	...

[a]** = significant at $P = 0.01$.

RESULTS AND DISCUSSION

To ascertain the source of possible differences in the samples as compared to the original study (Davis 1981) the T-RNV was run on the pea samples with and without defatting. Figure 1 shows the results observed in 1979 (Davis 1981) and in 1982 with and without defatting. Green pea products had significant differences associated with age of sample-plus-defatting, with processed form, and also a significant interaction between age of sample-plus-defatting and processed form, as shown in Table I. The interaction was primarily a result of the different response of the sterilized pea flour, which had a significant increase in T-RNV with age as compared to the rest of the processed forms, which had either a slight and insignificant loss in T-RNV, or a slight and insignificant increase in T-RNV. Defatting resulted in a significant increase in the T-RNV of the precooked powder as compared to the 1982 T-RNV. There was no significant effect on the green split pea or the

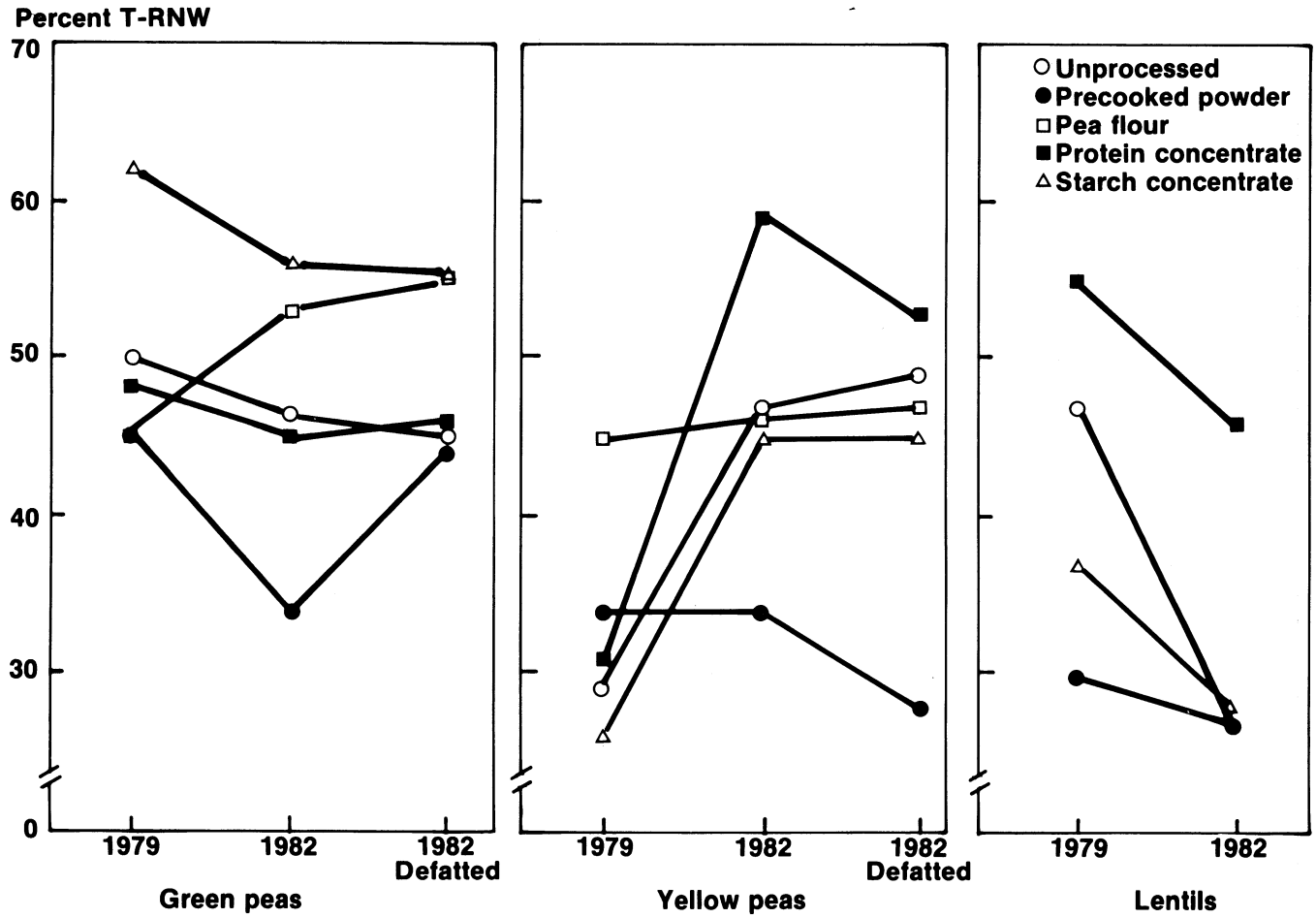

Fig. 1. *Tetrahymena*-relative nutritive value of processing forms of green peas, yellow peas, and lentils in 1979, 1982, and 1982 defatted.

other forms of green pea. Smith and Pena (1977) reported that a high concentration of chlorophyll inhibited the growth of *Tetrahymena*. The ether extraction of the green pea precooked powder may have decreased the chlorophyll, thus permitting a better growth response.

Yellow pea and processed forms showed an increase in T-RNV with age, though it was a small increase for the sterilized pea flour and the precooked pea powder. The protein concentrate, starch concentrate, and precooked pea powder had a decrease in T-RNV with defatting. In the previous study, Davis (1981) noted that the organisms grown on green and yellow pea products were two to three times larger than *Tetrahymena* grown on other substrates.

TABLE II
ANOVA of the Effects of Supplementation with Amino Acids, Variety of Legume, and Processed Form (excluding the flour) on T-RNV

Source	Degree of Freedom	Mean Squares[a]	Significant Difference[b,c]
Model	59	488.9**	
		0.948 R^2	
Legume type	2	4,774.5**	G,[a] Y,[b] L[c]
Processed form	3	1,153.3**	PC,[a] U,[b] SC,[c] P[d]
Type × process	6	775.7**	GSC,[a] GU,[a] GPC,[b] LPCC,[c] YU,[d] YPC,[d,e] YSC,[e,f] YP,[f,g] GP,[g] LU,[h] LP,[h] LSC,[i]
Supplement	4	1,506.8**	CM,[a] M,[b] CML,[c] C,[d] N,[e]
Type × supplement	8	78.5**	GCM,[a] GM,[a] GCML,[b] YCM,[c] GC,[c,d] YM,[c,d] YCML,[d] GN,[a] LCM,[e] YN,[e] LCML,[e] LM,[f] GC,[f] LC,[g] LU,[h]
Process × supplement	12	46.2**	PC-CM,[a] PC-M[a,b] U-CML,[c,d] U-M,[c,d] SC-M,[d] SC-CM,[d] PC-N,[e] SC-CML,[e] P-CM,[e,f] P-M,[e,f,g]
Type × process × supplement	24	67.0**	P-CML,[f,g,h] PC-C,[f,g,h] U-C,[g,h,i] SC-C,[h,i] SC-N,[i,j] U-N,[j] P-C,[j] P-N[k]
Error	120	12.1	
Total	179	...	

[a] ** = significant at $P = 0.01$.
[b] Determined by the least significant difference. Different superscript letters indicate that the means were significantly different.
[c] G = green, Y = yellow, L = lentil, U = unprocessed, P = precooked powder, F = sterilized flour, PC = protein concentrate, SC = starch concentrate, N = no supplement, C = cystine, M = methionine, and L = lysine.

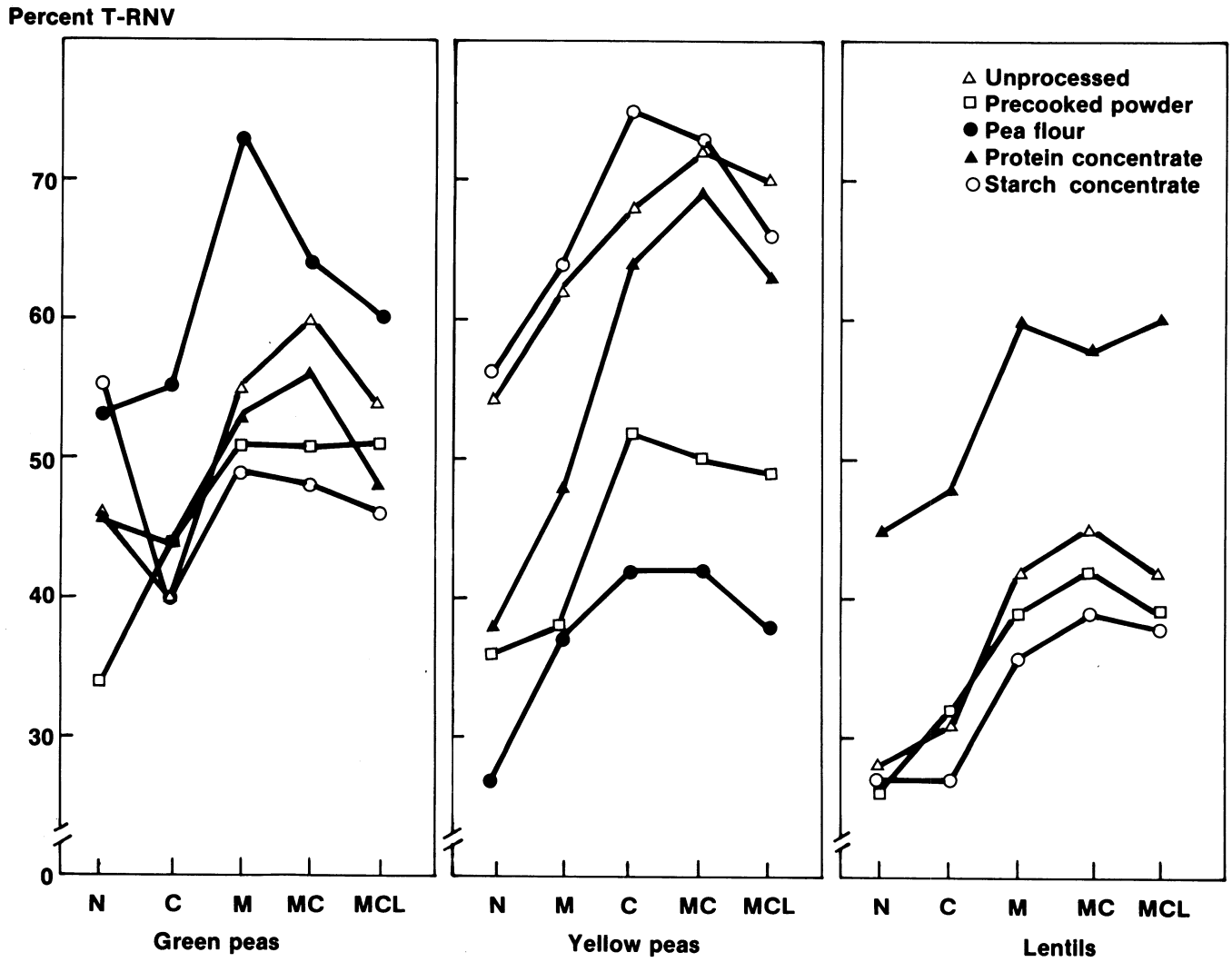

Fig. 2. Effect of supplementation with cystine, methionine, cystine + methionine, and cystine + methionine + lysine on *Tetrahymena*-relative nutritive value.

This large size was not apparent in 1982. There may be no difference in biomass produced between 1979 and 1982 trials. The literature contains little information about differences in protein quality of varieties of peas. One obscure report noted that there was no difference in average daily gain between pigs fed diets containing green, yellow, or black peas (Schneider and Christian 1951). The average daily gains for pigs consuming black, green, and yellow peas were 1.16, 1.24, and 1.29 lb per day, respectively, suggesting a trend. The main obvious difference between green and yellow peas is the presence of the green pigment in green peas. Based on that, green peas should give a lower T-RNV than yellow peas.

Lentils and processed forms of lentils all showed a decrease in protein quality with time, as shown in Fig. 1. The lentils and lentil processed products were not defatted. Tannins have been implicated in the inhibition of digestibility of both starches and proteins (Deshpande et al 1982, Ronnenkamp 1977, Mondragon and Gonzales 1978, Glick and Joslyn 1970). Davis (1981) suggested that the low T-RNV in the starch fraction of pin-milled and air-classified lentil was a result of the classification of tannin-containing seed coat into the starch concentrate. Sosulski (1979) suggested that the mechanism of depressed protein quality by tannins was a reaction with lysine or methionine. Schulz and Dumont (1977) reported that *Tetrahymena* has the ability to detoxify low levels of phenolics. In mammals, the detoxification of phenolics involves methylation. If *Tetrahymena* uses the same system, the added need for methyl groups would place an increased strain on the already limited methionine content of the legumes. All samples, except the split peas and the whole lentils, were stored in a finely ground form, and oxidation of various constituents may have occurred during storage.

If depressed T-RNV is due simply to complexation of methionine, cystine, and/or lysine, making these amino acids unavailable to the organism, then it should be possible to improve nutritive value by supplementation with appropriate amino acids. Supplementation of tannin-containing lentil products should result in a greater improvement of T-RNV than supplementation of green or yellow peas and pea products. Thus, these products were supplemented with cystine (C), methionine (M), cystine + methionine (CM), and cystine + methionine + lysine (CML). Lysine was added with methionine and cystine to overcome the "first limiting" status of the sulfur-containing amino acids so that the effect of supplemental lysine could be seen. Evans (1978) reported that up to 60% of the methionine nitrogen could be replaced by cystine nitrogen for *Tetrahymena* without depressing the growth of the organism, and that 2.89% of the total protein should be methionine + cystine for maximum growth response.

Figure 2 shows the response of *Tetrahymena* to the supplemental amino acids for each of the processed forms by type of legume. Analysis of variance in a factorial design, using a model that incorporated three types of legumes, four processes, five supplement levels, and the interactions of type and supplement, type and process, process and supplement, and process, type, and supplement accounted for 94.8% of the observed variability in T-RNV, as shown in Table II. Where there were significant differences with the analysis of variance, the LSD was used to determine which means were significantly different from each other. In all cases of significant differences, they were significant at the 0.0001 level.

There were significant differences by type, with each type being significantly different from the other two types. Types were ranked in decreasing order, G, Y, and L. Because there was no lentil flour, the flour process was not included in this model. There were significant differences by processing form, with each processing form being significantly different from all other processing forms. The forms were ranked in decreasing order as follows: PC, W, SC, and P. There were significant differences in supplements, with each supplement being significantly different from all other supplements. When averaged over all types and processed forms—whole, precooked powder, protein concentrate, and starch concentrate—the supplements were ranked in decreasing order as follows: CM, M, CML, C, and N. In green pea flour, which was not in this model, there was a significantly lower T-RNV, with MC as compared to M supplements. Other exceptions were of smaller magnitude. Lentil protein concentrate, yellow pea starch concentrate, and yellow pea precooked powder all showed a lower T-RNV with CM than with M supplements. This lower T-RNV indicates that the toxic level of sulfur-containing amino acids—120% of the requirement level—had probably been exceeded (Evans and Witty 1979). With the addition of CML only lentil protein concentrate had an increase in T-RNV as compared to CM supplements, and that was probably not a significant amount. The addition of lysine decreased growth on green peas, green pea-sterilized flour, and protein concentrate, all yellow pea products except the precooked powder, and all lentil products, except the protein concentrate, where only a slight improvement was observed.

Analysis of variance with a second model that incorporated two types (green and yellow peas), five processed forms, five supplements, and the interactions of type and processed form, type and supplement, processed form and supplement, and type, processed form, and supplement is shown in Table III. This model accounted for 93% of the observed variability. The significant differences in T-RNV were related to type of legume, and green was again ranked higher than yellow. There were significant differences due to processed form with the following ranking, in decreasing order, according to the LSD: W, SC, PC, F, and P. The difference in ranking in the two models is due to the higher T-RNV of the lentil starch concentrate compared to the other lentil forms—a relationship not seen in the other types of legume. There were significant differences in T-RNV due to supplement with the following (decreasing) ranking: M, MC, MCL, C, and N. The interactions were all significant; processing did not affect T-RNV the same way in all types of legume, and supplement did not affect T-RNV the same way in all types of legume or in all processed

TABLE III
ANOVA of the Effects of Processed Form and Amino Acid Supplement on the T-RNV of Green and Yellow Peas

Source	Degree of Freedom	Mean Squares[a]	Significant Difference[b,c]
Model	49	361.2**	
		0.93 R^2	
Legume type	1	540.0**	G,[a] Y,[b]
Processed form	4	696.0**	U,[a] SC,[a,b] PC,[b] F,[c] P[d]
Type × form	4	1,706.2**	GSC,[a] GU,[a] YF,[b] GPC,[b] YU,[c] YF,[c,d] YSC,[d] YP,[d,e] GSC,[e] GF,[f]
Supplement	4	1,045.8**	M,[a] CM,[a] CML,[b] C,[c] N[c]
Type × supplement	4	42.0*	GCM,[a] GM,[a] GCML,[b] YM,[b] YCM,[b] YCML,[c] GC,[c] YN,[d] GN,[d] YC,[d]
Form × supplement	16	63.6**	
Type × form × supplement	16	136.4**	
Error	100	13.9	
Total	149		

[a]** = significant at $P = 0.01$.
[b]Determined by the least significant difference. Different superscript letters indicate that the means were significantly different.
[c]G = green, Y = yellow, L = lentil, U = unprocessed, P = precooked powder, F = sterilized flour, PC = protein concentrate, SC = starch concentrate, N = no supplement, C = cystine, M = methionine, and L = lysine.

forms. In consideration of the apparent changes in protein quality that occurred with storage time, one cannot conclude that the organism would have responded in the same manner to supplementation of new or fresh products.

Supplementation with MCL did not improve the growth of *Tetrahymena* grown on high-tannin lentils any more than it improved the response to peas. Axtell (1976) reported that supplementing high-tannin sorghum with lysine did not improve biological value, even though lysine was the first limiting amino acid. The implications are threefold: that the supplements were unable to overcome the effect of the tannins in the lentils because the amount of lysine added was not in excess of the amount of tannins; or that the tannins were toxic to the organism; or that the effect was due to inhibition of digestion of peptides by binding of enzyme, substrate, or cofactor.

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[Received July 15, 1983. Revision received March 2, 1984. Accepted March 7, 1984]