Nutritional Improvement of Bread with Lysine and γ-Glutamyllysine

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To assess whether the dipeptide N^{ϵ}-(γ-L-glutamyl)-L-lysine (glutamyllysine) can serve as a nutritional source of lysine, we compared the growth of mice fed (a) an amino acid diet in which lysine was replaced by four dietary levels of glutamyllysine; (b) wheat gluten diets fortified with lysine; (c) a wheat bread based diet (10% protein) supplemented before feeding with lysine or glutamyllysine (0, 0.75, 1.50, 2.25, and 3% lysine hydrochloride equivalent in the final diet), not cobaked; and (d) bread diets baked with these levels of lysine or glutamyllysine. With the amino acid diet, the relative growth response to glutamyllysine was about half that of lysine. The effect of added lysine on the nutritional improvement of wheat gluten depended on both lysine and gluten concentrations in the diet. With 10 and 15% gluten, 0.37% lysine hydrochloride produced a marked increase in weight gain. Further increase in lysine hydrochloride to 0.75% proved somewhat detrimental to weight gain. Lysine hydrochloride addition improved growth at 20 and 25% gluten in the diet and did not prove detrimental at 0.75%. For whole bread, glutamyllysine served nearly as well as lysine to improve weight gain. The nutritive value of bread crust fortified or not was markedly less than that of crumb or whole bread. Other data showed that lysine or glutamyllysine at the highest level of fortification, 0.3%, improved the protein quality (PER) of crumb over that of either crust or whole bread, indicating a possible greater availability of the second-limiting amino acid, threonine, in crumb. These data and additional metabolic studies with [U-^{14}C]glutamyllysine suggest that glutamyllysine, cobaked or not, is digested in the kidneys and utilized in vivo as a source of lysine; it and related peptides merit further study as sources of lysine in low-lysine foods.

INTRODUCTION

Wheat gluten, the major protein in many baking formulations, is considered a poor-quality protein, primarily because it has insufficient amounts of two essential amino acids: lysine, the first-limiting amino acid, and threonine, the second-limiting one. To compensate for the poor quality of most cereal proteins such as gluten, the minimum recommended daily allowance (RDA) for these proteins has been set at 65 g, compared to 45 g for good-quality proteins such as casein (Hegsted, 1977).

As noted by Ziderman and Friedman (1985), during baking, the mixture of protein, carbohydrate, and water plus additives in dough is exposed to two distinct transformations. Desiccation of the surface on exposure to temperatures reaching 215 °C produces the crust. The crust encloses part of the dough in steam phase at approximately 100 °C, resulting in the formation of the crumb.

Because lysine's ε-amino group interacts with food constituents to make it nutritionally less available (Bjorck et al., 1983; Finley and Friedman, 1973; Friedman, 1977a, 1982; Geervani and Devi, 1986; Gumbmann et al., 1983; Tsen and Reddy, 1977; Tsen et al., 1977; Sherr et al., 1989), the baking process further reduces the dietary availability and utilization of lysine, especially in the crust, which makes up about 40% of the bread by weight (see Experimental Procedures). Many such interactions have been described including (a) reaction of the amino group with carbonyl groups of sugars and fatty acids to form Maillard browning products; (b) formation of cross-linked amino acids such as lanthionine, lysinoalanine, and glutamyllysine; (c) interaction with tannins and quinones; and (d) steric blocking of the action of digestive enzymes by newly introduced cross-links, as well as native ones such as disulfide bonds (Friedman, 1977b, 1982; Otterburn, 1989; Otterburn et al., 1977; Ziderman et al., 1989). Because these reactions of lysine with other dietary components may lead to protein damage and to the formation of physiologically active compounds, an important objective of food science and nutrition is to overcome these effects.

In principle, it is possible to enhance the nutritional quality of bread by either (a) creating new wheat varieties through plant genetic manipulations with improved protein quality characteristics (Johnson and Mattern, 1978); (b) covalent attachment of lysine residues to food proteins (Chan et al., 1979; Ikura et al., 1985; Iwami and Yasumoto, 1986); or (c) amino acid fortification (Betschart, 1978; Jansen, 1981). A major problem encountered when free lysine is used to fortify foods is that the added amino acid can itself participate in browning and other side reactions (Friedman and Molnar-Perl, 1990; Molnar-Perl and Friedman, 1990a,b; Oste and Friedman, 1990). Because ε-acyllysine derivatives are less susceptible to Maillard reactions than free lysine (Finot et al., 1978), the main objective of this study was to compare the effectiveness of lysine and glutamyllysine to serve as a nutritional source of lysine for mice fed bread crust, crumb, and whole bread cobaked with these amino acids. Since the ε-NH_2 group of N^{ϵ}-(γ-L-glutamyl)-L-lysine (glutamyllysine) is blocked in

$$NH_2C\overset{\epsilon}{H}_2CH_2CH_2CH_2CH(NH_2)COOH$$

L-lysine

$$HOOCCH(NH_2)CH_2C\overset{\gamma}{H}_2\text{-}CO\text{-}NH\text{-}C\overset{\epsilon}{H}_2CH_2CH_2CH_2CH\text{-}(NH_2)COOH$$

N^{ϵ}-(γ-L-glutamyl)-L-lysine

the form of an isopeptide bond with the γ-COOH group of glutamic acid, as illustrated, expectations were that it should also undergo less damage than lysine during baking.

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EXPERIMENTAL PROCEDURES

Materials. Unbleached, unbrominated, malted, and enriched white wheat flour (Mellow Judith) was obtained from Con Agra Inc., Oakland, CA. Fresh yeast was obtained every 2 weeks as a gift from Red Star Yeast, Oakland, CA. L-Lysine hydrochloride and commercial wheat gluten were obtained from U.S. Biochemical Corp., Cleveland, OH, and [U-^{14}C]-L-lysine came from Amersham, England. The other lysine compounds were synthesized as previously described (Finot et al., 1978). The fermentation chamber and the roller sheeting/loaf shaping machine were made by National Manufacturing Co., Lincoln, NE. The humidity in the chamber was monitored with a wet and dry bulb hygrometer made by Premium Instruments, Chicago, IL. The gas oven was an EZ by E. J. Chubbuck Co., Inc., Oakland, CA. Oven racks rotated during baking to maintain uniformity. The dough kneader was a Hobart Manufacturing Co Model C100 (Troy, OH). The pans were EKCO Baker's Secret, size 14.6 × 7.6 × 5.4 cm, with a nonstick coating, obtained in a local market.

Lysine derivatives, N^{ϵ}-acetyl-L-lysine, N^{ϵ}-formyl-L-lysine, and N^{ϵ}-benzylidene-L-lysine, were synthesized as previously described (Finot et al., 1978). N^{ϵ}-(γ-L-glutamyl)-L-lysine used for the feeding tests was synthesized according to the patent mentioned in the same paper. The synthesis of N^{α},N^{ϵ}-diformyl-L-lysine was adapted from that of N^{ϵ}-formyl-L-lysine by using an excess of acetic anhydride. Radioactive lysine derivatives were obtained by using uniformly labeled [U-^{14}C]-L-lysine. The specific activities were 0.38, 0.38, 0.38, and 0.89 mCi/mmol for [^{14}C]-L-lysine, N^{α}-formyl-[^{14}C]-L-lysine, N^{ϵ}-formyl-[^{14}C]-L-lysine, and N^{ϵ}-(γ-L-glutamyl)-L-lysine, respectively.

The purity of the lysine compounds listed in the tables was confirmed by previously described analytical procedures (Finot et al., 1978). In addition, ion-exchange chromatography of N^{ϵ}-(γ-L-glutamyl)-L-lysine on an amino acid analyzer (Friedman et al., 1979) produced a single peak eluting in the same position as methionine. The same peak resulted from chromatography of a commercial sample of the glutamyllysine obtained from Sigma (St. Louis, MO).

Methods. *Baking Experiments.* The recipe for one loaf of bread consisted of 183.1 g of flour, 106.8 g of water, 3.5 g of NaCl, 6.1 g of yeast, and lysine or glutamyllysine as applicable. The level of lysine fortification was as follows: L-lysine hydrochloride was added to equal 0, 0.75, 1.50, 2.25, and 3.0% of the protein in the flour. Glutamyllysine fortification levels were the molar equivalent of the lysine hydrochloride series. The water and flour were brought to 30 °C and maintained at this temperature in an incubator overnight. The salt, yeast, and lysine additives were solubilized in the water and then added to the flour. The mixture was kneaded in a Hobart mixer at speed 1 for 30 s and then at speed 2 for a total of 8 min. The dough was divided into workable balls, approximately three loaves per ball. The balls were placed in large stainless steel bowls, smooth side up, and placed in a fermentation chamber (37 °C, 90% humidity) for 45 min. The dough was then turned out on a board and divided into standard size loaves. The loaves were made into oval balls and degassed by running through the roller sheeting machine. Each sheet was rolled into a loaf, using pressure to exclude air bubbles. The loaves were placed in the pans, smooth side up, and returned to the fermentation chamber (37 °C, 90% humidity) for 45 min. They were baked for 35 min at 215 °C, removed from the oven and the pans, and left to cool on open racks. With an electric knife, two-thirds of the loaves were trimmed of crust. Crumb is the soft part of the bread produced during removal of the crust with the electric knife. Crust is the brown portion that contains a small fraction of the crumb that sticks to it during cutting. The color of the crusts supplemented with the highest concentration of glutamyllysine was lighter than of the corresponding crusts supplemented with lysine. Crumb, crust, and whole bread were lyophilized and ground in a Wiley mill with a 1-mm screen. The crust constituted about 40% of the total weight. The resulting flours were analyzed for nitrogen on an Erba Model 1400 automatic nitrogen analyzer.

Bioassays. Biological utilization of several lysine derivatives as nutritional sources of lysine was tested by a 14-day growth assay in mice (Swiss Webster strain, Simonsen Laboratories, Inc., Gilroy, CA) by using an amino acid diet (Table I) in which lysine was omitted, to be partly or fully replaced by the derivative to be tested as described for each experiment (Friedman and Gumbmann, 1981, 1988). Mice were housed singly or two per cage. The cages were polycarbonate with stainless steel wire tops and pine shavings for litter. Feed and water were provided ad libitum. The temperature of the animal room was 22 ± 1 °C, and humidity was maintained at 50 ± 10%. The light cycle was from 6:00 a.m. to 6:00 p.m. light and from 6:00 p.m. to 6:00 a.m. dark, as regulated by an automatic timer. Animals were assigned so that all treatment groups had nearly the same initial body weight.

Table I. Composition of Amino Acid Basal Diet

ingredient	%	ingredient	%
L-Ala	0.35	L-Trp	0.174
L-Arg·HCl	1.35	L-Thr	0.82
L-Asn	0.60	L-Val	0.82
L-Asp	0.35	Alphacel[a]	3.00
L-Glu	3.50	corn oil	8.00
Gly	2.33	cornstarch	20.00
L-His·HCl	0.41	dextrose	38.33
L-Ile	0.82	salts USP XIV[b]	5.00
L-Leu	1.11	sodium acetate	1.31
L-Lys·HCl	1.35	water (added)	5.00
L-Met	1.17	complete vitamin mixture[b]	2.00
L-Phe	1.51		
L-Pro	0.35	total	100.00
L-Ser	0.35		

[a] Nutritional Biochemical Corp., Cleveland, OH. [b] Friedman and Gumbmann (1988).

Table II. Composition of Protein Basal Diets

ingredient	amount, %	ingredient	amount, %
protein	variable, at the expense of cornstarch	dextrose	43.3
Alphacel	3.00	AIN mineral mixture[a]	5.00
corn oil	8.00	water (added)	5.00
cornstarch	(21.7), variable depending on protein level	complete vitamin mixture[a]	2.00
		total	100.0

[a] Friedman and Gumbmann (1988).

The effect of lysine fortification on the weight gain and protein efficiency ratio (PER), defined as weight gain/protein intake, of wheat gluten was examined by feeding mice 10, 15, 20, and 25% wheat gluten in the diet (Table II) fortified with 0, 0.37, and 0.75% lysine hydrochloride for 21 days (Friedman et al., 1987).

Bread (whole, crust, or crumb), with and without lysine or glutamyllysine fortification, was fed to weanling mice for 14 days as the sole source of protein in the diet (Table II). All diets contained 10% protein (N × 6.25) plus various levels of lysine hydrochloride or glutamyllysine either cobaked or not cobaked (supplemented at time of feeding).

Cholesterol Assays. At the end of the 14-day feeding study, serum samples obtained from the brachial artery by axillary space incision were analyzed for total cholesterol content by a modification of the Lieberman–Burchard reaction on a Technicon AutoAnalyzer II.

Metabolic Studies. These were carried out as described previously (Finot et al., 1978). The metabolic transit of the radioactive lysine derivatives was studied in rats (80–100 g) kept for 24 h in a metabolic cage designed to collect $^{14}CO_2$, urine, and feces, separately. The animals received by stomach tube, after 1 night of fasting, 5 μCi of each lysine derivative solubilized in 1 mL of water. The radioactivity excreted during the 24-h experiment was expressed in percent of the ingested dose.

Statistics. The relative potencies of the lysine derivatives as a nutritional source of lysine were calculated as the slope ratios or horizontal distances between the growth curves (weight

Table III. Utilization of Lysine Derivatives Compared to That of L-Lysine in Mice in an Amino Acid Diet[a]

rel dietary level L-lysine, %	body wt gain,[b] g					
	L-lysine	N^{ϵ}-(γ-L-glutamyl)-L-lysine	N^{ϵ}-acetyl-L-lysine	N^{ϵ}-formyl-L-lysine	N^{ϵ}-benzylidene-L-lysine	N^{α},N^{ϵ}-diformyl-L-lysine
300	11.7					
200	13.0					
100	13.8	10.5	6.3			
75	13.5	7.8				
50	12.2	5.5	4.0	4.0	10.2	0.8
25	5.8		3.2	1.5		
12.5	1.8	0.7				
6.25	0.3					
0	−1.5					
slope ratio to L-lysine		0.45	0.30			
95% CI[c]		(0.42, 0.50)	(0.25, 0.34)			

[a] Lysine derivatives were fed on an equal molar basis to L-lysine. [b] Six mice per group; initial body weight, 10.5 g. Weight gain at 14 days. [c] CI, confidence interval; values in parentheses show lower and upper limits.

gain after 14 days per unit of dietary concentration) for which linearity was approximated. Slope ratio and parallel line analyses of growth data were performed as described by Finney (1978) with SAS using the general linear model (GLM) procedure (SAS, 1987). Confidence intervals of potencies relative to lysine were estimated by using Fieller's theorem (Zerbe, 1978). Individual body weights for the generally six mice per diet group were used in the bioassay analyses. All comparisons are based on lysine equivalents.

For Table III, data for relative dietary levels of amino acid diets greater than 50% were deleted for the L-lysine diet (1.35% lysine hydrochloride) since it exceeded the linear portion of the response curve. The slope ratio analysis requires two assumptions: linearity and common intercepts. The test for curvature is nonsignificant, $P = 0.22$, but the test for departure from common intercepts is significant, $P = 0.002$. Despite this violation of assumption, the slope ratio estimates still provide useful approximations of potencies in this case since the reduction in R^2 (coefficient of determination) is only from 0.937 to 0.926 from forcing the common intercept. (In looking at the graph, the potencies will be slightly underestimated.) The most useful information in the output are the slopes and standard errors and the potency ratios and their 95% confidence intervals.

The data in Tables V–VII and IX were subjected to standard parallel line analyses. In each case, the tests for lack of parallelism are nonsignificant ($P < 0.10$). For this type of assay, the potencies represent the distances between lines in the abscissa direction (concentration or percent gluten for Table IX).

RESULTS AND DISCUSSION

Utilization of Lysine Derivatives in Mice. To assess the potential value of several lysine derivatives as nutritional sources of lysine, growth response to N^{ϵ}-acetyl-, N^{ϵ}-benzylidene, N^{α}, N^{ϵ}-diformyl-, and glutamyllysine was determined in mice. Dose–response data were obtained as permitted by the supply of test material on hand. Table III shows that benzylidenelysine produced the greatest weight gain after lysine, followed by glutamyllysine. Acetyl- and formyllysines were equivalent at a relative concentration of about 30–50%, depending on the method used to calculate potency; however, multilevel feeding of formyllysine was not done. Diformyllysine barely supported growth.

The data are best visualized when plotted as shown in Figure 1. The dose–response curves show that relative responses varied with the dietary concentrations. It is also possible to rank the amino acid derivatives according to a relative growth response. This was defined as follows: percent relative weight gain equals the net growth response between 0 and 50% relative concentration of amino acid in the diet (Figure 1) divided by the growth response of L-lysine times 100. The calculated values in Table IV show a range of values from 16.8 for diformyllysine to 85.4% for benzylidenelysine, approximately of the same order as observed with rats (Finot et al., 1978). Note that glutamyllysine, the main object of our study, had a lysine equivalent value of 51.1%. Thus, any assessment of the relative damaging effects of baking on lysine or glutamyllysine should take into account the relative potencies of these two compounds in the amino acid diets. Note also that the slope ratio statistical analysis gave only a 45% lysine equivalent for the lysine part in glutamyllysine. The corresponding value for N^{ϵ}-acetyllysine was 30%.

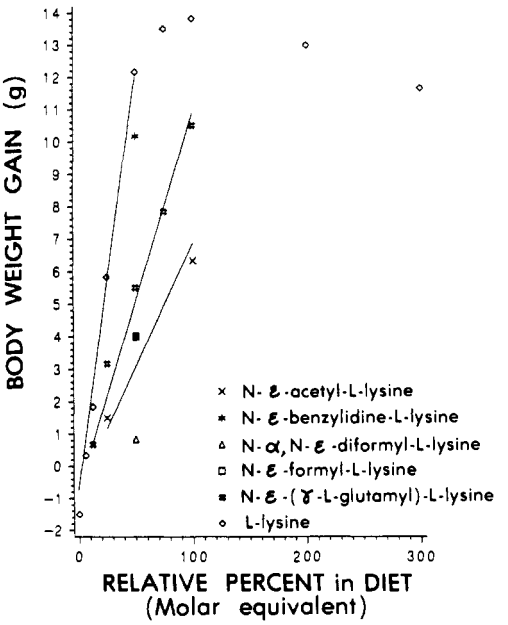

Figure 1. Weight gain in mice fed increasing dietary levels of lysine and lysine derivatives as part of an amino acid diet.

Table IV. Relative Growth Response to L-Lysine Derivatives in Mice[a]

amino acid	rel wt gain,[b] %	slope ratio analysis,[c] %
L-lysine hydrochloride	100.0	
N^{ϵ}-benzylidene-L-lysine	85.4	
N^{ϵ}-(γ-L-glutamyl)-L-lysine	51.1	45
N^{ϵ}-acetyl-L-lysine	40.0	30
N^{ϵ}-formyl-L-lysine	40.0	
N^{α},N^{ϵ}-diformyl-L-lysine	16.8	

[a] Comparison made at 50% relative concentration in the diet, which is equal molar to 0.68% L-lysine hydrochloride. [b] Percent relative weight gain equals the net growth response between 0 and 50% relative concentration of amino acid in the diet divided by that of L-lysine hydrochloride times 100. [c] Percent weight gain equals slope ratio to L-lysine (Table III) times 100.

Table V. Weight Gain and PER in Mice Fed Whole Bread with L-Lysine or Glutamyllysine Supplementation in the Diet (Not Cobaked)

added amino acid concn,[a] %	wt gain,[b] g		PER[c]	
	lysine	glutamyl-lysine	lysine	glutamyl-lysine
0	5.0	5.0	1.0	1.0
0.075	7.8	6.2	1.4	1.0
0.150	8.8	6.7	1.6	1.4
0.225	9.7	9.3	1.8	1.8
0.300	11.0	9.5	1.9	1.8
SE		±0.7	±0.3	
potency difference[d]		0.069		
95% CI		(0.026, 0.114)		

[a] L-Lysine hydrochloride concentration in the diet as shown; glutamyllysine added to achieve molar equivalent levels (factor = 275.3/182.6 = 1.51 × lysine concentration). [b] Mean weight gain at 14 days, six mice per group. [c] PER based on two mice per cage, $N = 3$. [d] Potency difference = added % glutamyllysine concentration required to achieve equivalent weight gain as with L-lysine.

A surprising result is that the lysine equivalent value of glutamyllysine is about 45–50% when evaluated as part of an all amino acid diet (Table IV) but nearly 100% when the dipeptide is added to diets containing wheat gluten (Tables V–VII). A possible explanation is that provision of maintenance lysine levels by gluten in the bread-based diets tends to reduce immediate need for lysine derived from added glutamyllysine. Since glutamyllysine has to reach the rat kidneys before it can be hydrolyzed to lysine, its utilization is delayed about 2 h compared to free or protein-bound lysine. There is an apparent difference in the bioavailability of lysine from the two different diets. A more likely explanation is that, because glutamyllysine is transported across the intestinal tract by passive transport which is disturbed by the high osmolarity of the free amino acids in the amino acid diets, its absorption is reduced compared to that in glutamyllysine-fortified protein diets (Reichl, 1989; Scharrer, 1989).

Metabolic Studies. The metabolic fates of the lysine derivatives are relevant to their possible nutritional value and safety. Preliminary indications (Finot et al., 1978; Hurrell and Carpenter, 1978) suggest that the derivatives go through metabolic pathways different from those followed by lysine. The following are some additional findings in rats. L-Lysine, N^{α}-formyl-L-lysine, N^{ϵ}-formyl-L-lysine, glutamyllysine, all labeled with [^{14}C]lysine, were fed to rats by stomach tube. The resulting $^{14}CO_2$, urine, and feces were collected for 24 h in metabolic cages. The N^{α}-formyllysine, which is not utilized as a source of lysine, was mainly excreted in the urine (Table VIII), showing that this molecule was not deformylated by the kidneys. The N^{ϵ}-formyllysine, which is 50% utilized as a source of lysine, was also mainly excreted in the urine. Evidently, this molecule cannot be completely deformylated by the kidney. In contrast, glutamyllysine, which is a good source of lysine, was not excreted in the urine. The low level of digestibility as measured by the high level of radioactivity (35.7%) in the feces is probably due to route of administration through tubing inserted into an empty stomach. When given in a diet for several days in a bioavailability trial, this molecule was more efficiently absorbed by the gut than when it was given by stomach tubing in fasted animals.

The incorporation of the labeled compounds into intestinal mucosa, liver, and kidney tissues 24 h after ingestion was found to be the same for both lysine and glutamyllysine. In contrast, when the two compounds were administered intravenously, the $^{14}CO_2$ peak in the expired air appeared approximately between 165 and 195 min after administration of glutamyllysine and only after about 30 min following injections of lysine. These and additional studies with tissue homogenates suggest that glutamyllysine has to reach the kidneys before it is hydrolyzed to lysine.

The $^{14}CO_2$ comes from the catabolism of lysine in the gut and from its catabolism in the body. The N^{α}-formyl-[^{14}C]lysine was found to be excreted in the urine as N^{α}-formyllysine only. This molecule was not transformed in the body.

N^{ϵ}-Formyl[^{14}C]lysine was detected in traces in the urine. The main radioactive product found in the urine had an acidic behavior on ion-exchange chromatography and on paper electrophoresis. On acid hydrolysis, this molecule regenerated lysine. The main urinary catabolite is probably N^{ϵ}-formyl-N^{α}-acetyllysine, formed by the kidney to detoxify N^{ϵ}-formyl-L-lysine by acylation of the α-amino group.

These metabolic studies show that the bioavailability of the acyl derivatives of lysine depends on the absorption rates (passive diffusion mechanism) by the intestine and by the capacity of the kidneys to liberate lysine. The kidneys have the ability to liberate ϵ- but not α-amino groups from acylated lysine derivatives.

L-Lysine Fortification of Wheat Gluten. In addition to the already mentioned studies on the utilization of lysine derivatives as part of an all amino acid diet, we studied the availability of lysine when added to wheat gluten (Table IX; Figures 2 and 3) to design quantitative studies of the effect of fortifying bread with lysine (Table III).

As expected, weight gain and PER were improved by the addition of lysine. The effect of any given level of added lysine, however, depended on the level of gluten in the diet. With 10 and 15% gluten, 0.37% lysine produced a marked increase in weight gain and improvement in protein quality as measured by PER (Figures 2 and 3). Further increase in lysine to 0.75% proved to be somewhat detrimental. Gluten at 20 and 25% in the diet produced better growth than gluten at 10 or 15% without lysine supplementation. Lysine addition improved growth at the higher gluten levels and surprisingly did not prove detrimental at 0.75%. At lower gluten levels, the ratio of added lysine to total protein at 0.75% lysine apparently unbalanced the essential amino acids, causing one other than lysine to become nutritionally limiting and thereby reducing protein nutritional quality and growth.

The absolute PER of gluten was markedly superior at 10% gluten plus 0.37% lysine than at higher levels of gluten without supplementation (Figure 3). Except for unsupplemented gluten, increasing the amount of gluten in the diet decreased PER; i.e., less growth occurred per unit of protein eaten. This is a well-known characteristic of protein nutrition (Owens and Pettigrew, 1989): after sufficient protein is supplied to meet basic demands, increased amounts of protein allow the animal to use it for purposes other than growth, thus decreasing the efficiency of utilization for growth (PER).

Another interesting observation is that plots of body weight gain versus percent of gluten in the diet supplemented with 0, 0.37, or 0.75% lysine hydrochloride produced two parallel lines (Figure 3). These plots show (a) a linear increase in weight gain with gluten levels in the diet ranging from 10 to 25%, (b) a dramatic enhancement in weight gain with the gluten diets containing added 0.37% lysine hydrochloride, and (c) weight gains with the 0.37% lysine hydrochloride diets were essentially the same as with the 0.75% lysine diets (Table IX).

These phenomena merit further analysis, first in terms of the amino acid composition of gluten and the nutritional requirements of mice. For gluten, lysine is the first-limiting amino acid (N = 1.4 g/16 g of N) and threonine

Table VI. Weight Gain and PER in Mice Fed Whole Bread, Bread Crumb, and Bread Crust Supplemented with Lysine before and after Baking

lysine hydrochloride concn, %	wt gain,[a] g			PER[a]		
	whole bread	bread crumb	bread crust	whole bread	bread crumb	bread crust
			Cobaked			
0	4.0	4.5	2.7	0.9	0.9	0.5
0.075	6.5	8.8	2.8	1.3	1.7	0.6
0.150	7.7	9.8	4.0	1.4	2.1	0.9
0.225	9.3	9.7	5.8	1.8	1.9	1.1
0.300	8.8	10.2	6.5	1.6	1.9	1.3
SE		±0.6			±0.1	
potency difference[b]		−0.085	0.185			
95% CI		(−0.148, −0.029)	(0.124, 0.263)			
			Not Cobaked			
0	5.0	6.3	2.2	1.0	1.2	0.6
0.075	7.8	8.8	2.8	1.4	1.9	0.7
0.150	8.8	9.5	6.0	1.6	1.9	1.5
0.225	9.7	9.8	6.7	1.8	2.2	1.4
0.300	11.0	9.8	7.7	1.9	3.3	1.7
SE		±0.7			±0.3	
potency difference		−0.055	0.207			
95% CI		(−0.135, 0.016)	(0.128, 0.318)			

[a] Mean weight gain at 14 days, six mice per group. Casein control = 12.0 g. [b] Potency difference × 100 = % additional lysine hydrochloride needed to achieve weight gain as with whole bread.

Table VII. Weight Gain and PER in Mice Fed Whole Bread, Bread Crust, and Bread Crumb Cobaked with Glutamyllysine[a]

glutamyllysine concn (lysine equivalent), %	whole bread	bread crumb	bread crust
		Weight Gain	
0	5.2	7.5	3.0
0.075	6.5	9.0	3.5
0.150	7.3	9.8	3.7
0.225	8.5	9.7	3.7
0.300	9.8	9.5	5.8
SE		±0.5	
potency difference[b]		−0.201	0.376
95% CI		(−0.325, −0.116)	(0.265, 0.560)
		PER	
0	0.92	1.2	0.6
0.075	1.3	1.6	0.6
0.150	1.2	1.6	0.7
0.225	1.3	1.6	0.6
0.300	1.5	1.7	1.0
SE		±0.1	

[a] Mean PER at 14 days, two mice per cage, $N = 3$. Mean weight gain at 14 days, six mice per group. [b] Potency difference × 100 = % additional glutamyllysine needed to achieve weight gain as with whole bread.

Table VIII. Percent of Radioactivity Excreted in CO_2 Urine and Feces 24 h after Intragastric Ingestion of [U-^{14}C]Lysine Derivatives (Rat Studies)

	lysine ($n = 2$)	N^{ϵ}-L-formyl-L-lysine ($n = 4$)	N^{α}-formyl-L-lysine ($n = 2$)	N^{ϵ}-(γ-L-glutamyl)-L-lysine ($n = 2$)
CO_2	18.5 ± 0.9	17.3 ± 2.1	12.7 ± 3.0	28.0 ± 4.0
urine	5.5 ± 0.6	25.1 ± 2.2	73.6 ± 3.6	2.75 ± 0.5
feces	7.6 ± 1.1	3.2 ± 0.6	5.6 ± 0.8	35.7 ± 10.0

the second ($N \sim 2.5$ g/16 g of N). For mice, the minimum lysine and threonine requirements are about 0.4% each in the diet (Bell and John, 1981). If we calculate the levels of lysine and threonine in the diets, it can be shown that lysine is limiting in only a few diets (10% gluten diets, for example) and that threonine is limiting only in diets containing about 10–15% gluten, as illustrated in Table X. These considerations suggest that no improvement in growth would be expected for diets fortified with 0.37 and 0.75% lysine hydrochloride because lysine is not limiting.

Table IX. Effect of Unsupplemented and Lysine-Supplemented Gluten on Weight Gain (Grams) in Mice after 21 Days

gluten, %	lysine, %		
	0	0.37	0.75
10	3.5	11.3	10.5
15	6.2	15.5	15.2
20	9.7	17.5	18.7
25	12.8	19.0	19.3
potency difference from 0 lysine		13.5	13.7
95% CI		(10.6,17.3)	(19.7,17.4)

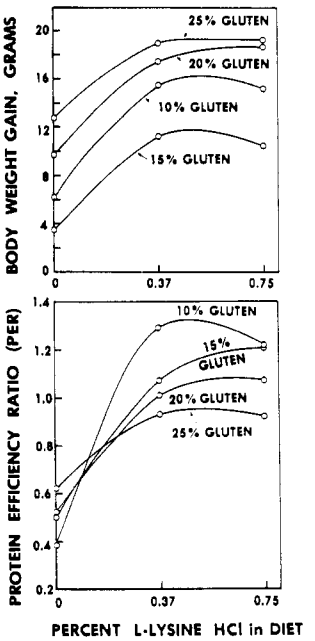

Figure 2. Effect of gluten and lysine in diets on body weight gain and PER in mice after 21 days.

Second, amino acids are used both metabolically as building blocks for protein biosynthesis and catabolically as energy sources. Catabolism for most amino acids proceeds through transamination pathways; the exceptions

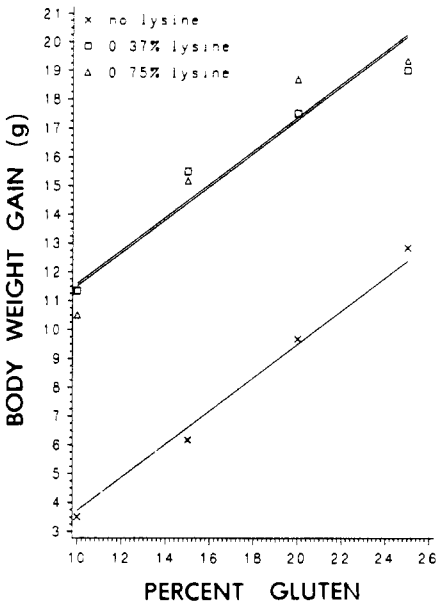

Figure 3. Plots of body weight gain in mice after 21 days as a percent of gluten supplemented with 0, 0.37, or 0.75% lysine hydrochloride.

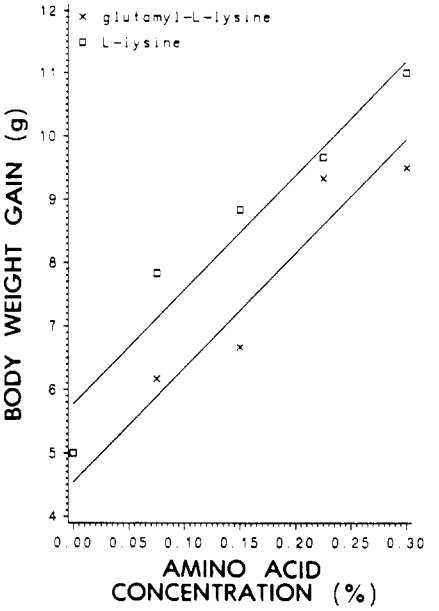

Figure 4. Weight gain in mice after 14 days fed whole bread supplemented with L-lysine or glutamyllysine in the diet (not cobaked, Table V).

Table X. Calculated Nutritionally Limiting Amino Acids as a Function of Gluten and Lysine Concentration[a]

	no lysine added		0.37% lysine hydrochloride added		0.75% lysine hydrochloride added	
gluten, %	lysine, %	threonine, %	lysine, %	threonine, %	lysine, %	threonine, %
10	0.14[b]	0.25[c]	0.44	0.25[c]	0.74	0.25[c]
15	0.21[b]	0.37[c]	0.51	0.37[c]	0.81	0.37[c]
20	0.28[b]	0.50	0.58	0.50	0.88	0.50
25	0.35[b]	0.62	0.65	0.62	0.95	0.62

[a] 0.4% lysine and threonine is required by mice (Bell, 1981). [b] Limiting in lysine. [c] Limiting in threonine.

are lysine and threonine (Lougnon and Kiener, 1989; Khan-Siddiqui, 1989; Milner, 1989). These two amino acids (which are nutritionally limiting in wheat gluten) are catabolized by non-aminotransferase-specific enzymes: threonine dehydratase acts on threonine and lysine ketoglutarate reductase on lysine (Hegsted, 1977).

Chu and Hegsted (1976) showed that the concentrations of these enzymes in the liver of rats are subject to adaptive responses that control the utilization of these two amino acids. Although both enzymes are induced by feeding diets high in protein, rats differ in the mechanism of the adaptive response to high-protein diets and to diets whose threonine or lysine content is less than needed for growth. Thus, reductase falls to very low levels in the liver of rats fed wheat gluten. This appears to be an adaptive response conserving body lysine. At the same time catabolism of body proteins increases, producing endogenous lysine needed for survival. These considerations imply that as the level of wheat gluten in the diet decreases, lysine is no longer the limiting amino acid. Total protein or some other amino acid then becomes limiting.

In contrast to the apparent mechanism of lysine catabolism, Chu and Hegsted (1976) report that threonine dehydratase does not appear to be substrate induced. It appears, therefore, that when lysine is the limiting amino acid, the catabolic enzyme falls to low levels and lysine is conserved at the expense of body proteins. Loss of tissue proteins is much less when a diet low in threonine is fed, since the level of threonine dehydratase does not seem to be significantly affected by the protein or threonine content of the diet. Additional studies are needed to establish whether the catabolic enzyme patterns in mice parallel those of rats.

Fortification of Wheat Bread with Lysine and Glutamyllysine after Baking. Tables V and VI compare stimulation of growth and effect on PER by lysine and glutamyllysine added after baking (not cobaked). The results suggest that glutamyllysine was slightly less effective than lysine, although differences were not statistically significant. Plotting the data (Figure 4) reveals that mice gained somewhat more weight from eating whole bread supplemented by lysine (not cobaked) than by glutamyllysine. The relative potencies of the two compounds in improving the diet were the same, as shown by weight gain per unit added amino acid. That is, the slopes of the two growth curves were equal after initial supplementation.

Table VI shows that crumb produced slightly greater weight gain than did whole bread at each lysine level added. The differences were not statistically significant. The weight gain with crumb at the highest level of lysine was 82% that of casein. Crust, as expected, was associated with reduced growth. This reduction could be overcome to a large degree by lysine supplementation. Whether greater levels of lysine than those tested increase growth for crust to that produced by crumb is not known. The corresponding experiment with glutamyllysine was not done.

Figures 5–7 reveal that the protein quality of crust was less than that of whole bread, but differences were not significant mainly due to the increased variation associated with feed consumption data used in calculating weight gain. Thus, the PER calculation in mice has greater error than that of weight gain. Lysine supplementation improved protein quality of both crust and whole bread. At the highest level added, 0.3%, the PER of crumb was significantly greater than that of crust and whole bread. This may indicate greater digestibility and availability of other limiting amino acids in crumb and, thus, the greater potential for improvement in crumb when the first-limiting amino acid, lysine, is supplemented.

Fortification of Wheat Flour with Lysine before Baking. To establish the effect of baking on lysine utilization, wheat flour was fortified with lysine at the following levels: 0, 0.75, 1.50, 2.25, and 3.0% of flour protein. This flour was then baked into bread. Part of the bread was separated into crumb and crust, freeze-

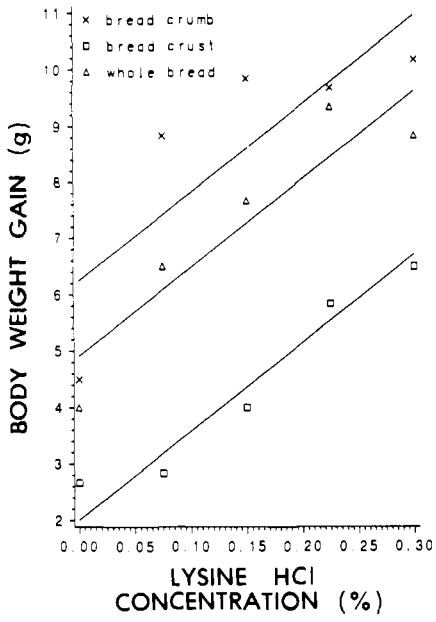

Figure 5. Weight gain in mice after 14 days fed whole bread, bread crumb, and bread crust supplemented with lysine before baking (cobaked, Table VI).

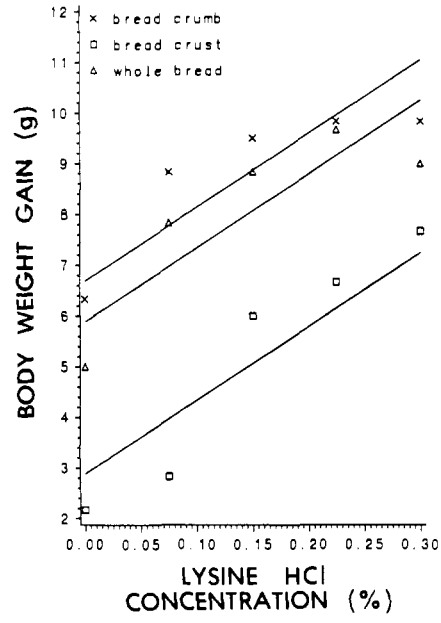

Figure 6. Weight gain in mice after 14 days fed whole bread, bread crumb, and bread crust supplemented with lysine (not cobaked, Table VI).

dried, and milled into a baked flour. The whole bread, crust, and crumb were then fed to mice in a 14-day growth assay in which diets contained 10% protein provided by the bread samples.

As with lysine-supplemented diets (not cobaked), addition of lysine to flour before baking (cobaked) also greatly improved the nutritional quality of the wheat protein. Table VI shows that both weight gain and protein quality (PER) were significantly increased. Supplementation had similar effects on weight gain whether cobaked or not. Maximum growth with crumb could be achieved with approximately 0.3% lysine, whereas crust might possibly be improved further with even greater lysine additions.

Figure 5 shows that improvement of protein quality of crumb through flour supplementation (cobaked) seemed to level off at less than 0.3% lysine. Lysine at 0.3% produced no further improvement, if not actual loss in

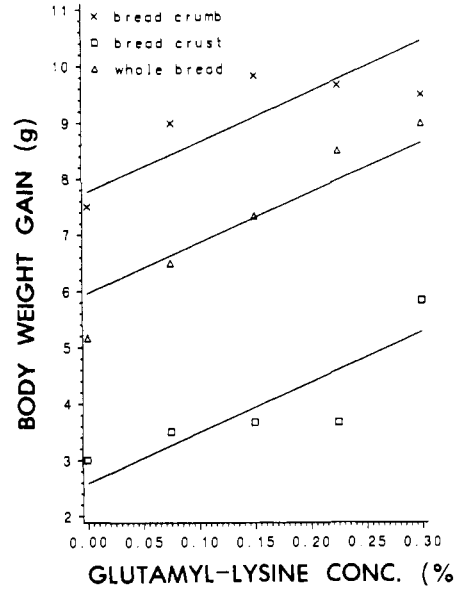

Figure 7. Weight gain in mice fed whole bread, bread crumb, and bread crust cobaked with glutamyllysine (Table VII).

protein quality at the higher level. Perhaps accelerated browning at higher lysine concentrations was responsible for this limit on the beneficial effect of lysine addition before baking.

Crust, as expected, had distinctly lower nutritive value than either crumb or whole bread. This suggests that crust relative to crumb is additionally deficient in lysine as a result of its greater exposure to heating during baking and, with sufficient lysine supplementation, may be improved close to the nutritional level of crumb. As already mentioned, further improvement of crust might be expected with greater lysine supplementation than included in this assay, whereas that of the crumb and whole bread appeared to reach maximum.

Fortification of Wheat Flour with Glutamyllysine before Baking. The protocol of this assay was the same as that described in which lysine was used to fortify wheat flour before bread baking. The fortification with glutamyllysine was on a percent equivalent to lysine on a molar basis relative to protein content.

Table VII reports body weight gain and PER. Glutamyllysine cobaked with wheat flour significantly stimulated growth of mice fed the bread preparations as the sole source of dietary protein and improved the quality of wheat protein. The relative nutritive values of crumb, crust, and whole bread are similar to those observed with lysine, crust being the most heat damaged. As with cobaked lysine, maximum improvement occurred near 0.3% glutamyllysine.

Comparison of Lysine and Glutamyllysine Added before Baking. Tables V–VII and Figures 4–7 compare the effect of lysine to that of glutamyllysine on weight gain and PER. When these amino acids were not cobaked with the bread diet, lysine was slightly more potent than glutamyllysine in stimulating growth and improving PER. This is still the case when the amino acids were not cobaked with the flour in the simple bread formulation. With regard to weight gain (Figures 5 and 6), the overall effect of the two amino acids cobaked with flour was similar. Except for crust, both resulted in maximum growth at the 0.3% lysine equivalent level.

Protein quality (PER) improvement with lysine supplementation showed an apparent maximum at levels below 0.3% for both crumb and whole bread, whereas continued improvement of crust at levels greater than 0.3% seemed

possible. For glutamyllysine, however, a maximum effect for crust at these levels was not apparent.

A statistical analysis of variance was run for weight gain and PER. Significant effects on weight gain and PER were associated with type of bread preparation (crumb, crust, and whole bread) and level of amino acid supplementation ($P < 0.0001$). Parallel line model analyses of variance were used for the entire sets of data represented by the figures. Overall, the two forms of lysine did not produce significantly different weight gains. Protein quality (PER), however, was improved to a greater extent by lysine ($P < 0.005$). Since Figures 5 and 7 represent separate experiments, the results can only be compared with regard to relative potencies among crumb, crust, and the whole bread.

In matching the two assays by analysis of variance, it was found that bread preparations with no amino acid supplementation (0% supplementation) resulted in overall PERs that were not significantly different. Thus, essentially the same protein quality for unsupplemented wheat proteins was obtained in both assays. In the two experiments, absolute weight gain was not the same, however. Weight gain was consistently greater for mice in the glutamyllysine run with each unsupplemented bread preparation (overall, $P < 0.0006$). One large assay with one lot of mice fed all diets simultaneously would be ideal to overcome this problem, but would be difficult to conduct.

Nutritional Significance. Several considerations relevant to the theme of this study will be briefly summarized to help place our findings into proper perspective.

(1) γ-Glutamyllysine cross-links are also formed in vivo in various tissues by the catalytic action of transglutaminases (Fink et al., 1980). These authors also report the partial purification and characterization of γ-glutamylcyclotransferase from rabbit kidneys, for which glutamyllysine is a substrate. Normal hydrolytic enzymes such as pepsin or trypsin do not cleave the isopeptide bond of glutamyllysine (Loewy, 1984).

(2) Raczynski et al. (1975) examined the metabolism of tritium-labeled glutamyllysine in the rat. They report that the compound is largely absorbed unchanged from the intestine and metabolized. This observation and the observed findings with tissue homogenates show that hydrolysis of the isopeptide bond of glutamyllysine largely takes place in the kidney.

(3) Waibel and Carpenter (1972) found that the growth-promoting activity of glutamyllysine supplementation to low-lysine diets was nearly equivalent to that of L-lysine for both young rats and chicks. In related studies, Hurrell and Carpenter (1977) and Otterburn et al. (1977) report that heated food proteins containing isopeptide bonds are utilized less efficiently than unheated starting materials. This loss in protein quality may be due to reduced protein digestibility arising from steric hindrance of the introduced cross-links to the action of proteolytic enzymes in the digestive tract.

(4) Other studies have successfully introduced lysine residues into low-lysine proteins such as gluten through (a) transglutaminase-catalyzed attachment of lysine residues to food proteins (Iwami and Yasumoto, 1986), (b) transglutaminase-catalyzed incorporation of lysyl dipeptides into food proteins (Ikura et al., 1985), and (c) carbodiimide-mediated covalent attachment of lysine, N^{ϵ}-acetyllysine, or N^{ϵ}-benzylidenelysine (Chan and Nakai, 1981). Although these approaches seem effective in improving the nutritional quality of food proteins, as well as their resistance to browning, they have disadvantages over the direct fortification described in the current study. These include (a) possible side reactions involving transglutaminase-catalyzed cross-linking of protein chains (Kurth and Rogers, 1984; Motoki and Nio, 1983) and (b) the high cost of glutaminase and uncertainty about the safety of the chemical treatments.

Table XI. Plasma Cholesterol in Mice Fed Whole Bread, Bread Crumb, and Bread Crust with Lysine or Glutamyllysine Supplementation

	plasma cholesterol[a]			
diet	−L-lysine	+lysine[b]	−glutamyl-lysine	+glutamyl-lysine[c]
		Not Cobaked		
whole bread	101[c]	140[ab]		152[a]
bread crumb	112[bc]	127[abc]		
bread crust	111[bc]	123[abc]		
casein control		131[abc]		
SE		±10		
		Cobaked		
whole bread	89[c]	133[ab]	102[c]	131[ab]
bread crumb	93[c]	135[a]	100[c]	136[ab]
bread crust	94[c]	103[bc]	108[bc]	133[a]
casein control	129[ab]		124[ab]	
SE		±10	±7.2	

[a] Mean plasma cholesterol (mg/100 mL) at 14 days, six mice per group. Means without a superscript letter in common for a given baking procedure are significantly differently, $P < 0.05$, Duncan's multiple range test. [b] L-Lysine hydrochloride concentration in the diet was 0.3%. [c] Glutamyllysine was equimolar to 0.3% L-lysine hydrochloride.

(5) In addition to catalyzing the formation of glutamyllysine, heat and alkali treatments of food proteins also induce the concurrent formation of N^{ϵ}-(DL-2-carboxyethyl)-L-lysine or lysinoalanine. In contrast to the described high utilization of glutamyllysine as a nutritional source of lysine, the biological utilization of lysinoalanine, determined in a growth assay in weanling male mice in which all L-lysine in a synthetic amino acid diet was replaced by a molar equivalent of lysinoalanine, produced a weight gain equivalent to that expected from diet containing 0.05% L-lysine (Friedman et al., 1982, 1984). Evidently, acyllysine derivatives such as glutamyllysine containing isopeptide bonds are hydrolyzed by kidney enzymes to lysine to a greater extent than alkyllysine derivatives such as lysinoalanine (de Weck-Gaudard et al., 1988).

(6) In related studies we describe chemical transformation of gluten during baking, at high pH, and in the presence of vinyl compounds, ultraviolet radiation, and ninhydrin (Friedman et al., 1970, 1984a,b; 1990; Cavins and Friedman, 1967, 1968; Krull and Friedman, 1967; Eskins and Friedman, 1970a,b).

Plasma Cholesterol. Total plasma cholesterol sampled at the end of the 14-day feeding period was determined in mice fed bread and casein diets with and without supplementation by lysine and glutamyllysine (Table XI). Cholesterol levels were not significantly different among mice fed any of the bread diets. Increased cholesterol level in mice fed lysine- and glutamyllysine-fortified bread may be due to the nutritional improvement of the diet alone or to other factors. It is generally recognized that plasma cholesterol is depressed in mice fed diets poor in protein or amino acids (Sanchez and Hubbard, 1989) and that increases in the dietary lysine/arginine ratio increase cholesterol levels (Kritchevsy, 1989).

CONCLUSIONS

Our studies in mice complement previous studies in rats and chicks and suggest that glutamyllysine is hydrolyzed in the kidneys to lysine. The peptide is utilized more efficiently as a source of lysine when fed as part of a baking formulation than it is when fed as part of an all amino acid

diet. Such lysine peptides merit further study as a potential source of lysine for animals and humans consuming low-lysine foods (Sarwar and Paquet, 1989; Sarwar et al., 1985). Our results also show that mice provide a good animal model to study protein quality of native, fortified, and processed wheat proteins (Cossak and Weber, 1983). Mouse bioassays have a major advantage especially useful in applications to label foods for protein nutritional quality. They require about one-fifth of the test material compared to the rat animal model. Finally, the present study should be extended to other baking formulations that may be more susceptible to browning than the simple formula used here consisting of wheat flour, yeast, and water.

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