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COVALENT ATTACHMENT OF LIMITING AMINO ACIDS
TO WHEAT GLUTEN
FOR NUTRITIONAL IMPROVEMENT

by

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ABSTRACT

The benefits of fortification of poor quality food proteins such as wheat gluten with limiting amino acids depend on the biological availability of the added amino acids and their stability with respect to processing and storage. Although simple addition of amino acids in free form is convenient, the potential improvement in nutritional quality by this method of fortification may not materialize due to possible losses during processing steps such as washing, susceptibility to degradative reactions, and different rates of absorption and utilization compared to protein-bound amino acids.

In this study, covalent attachment of lysine and threonine to wheat gluten was investigated using the chemical carbodiimide reaction and the enzymatic plastein reaction. The nutritional quality of enriched products was evaluated by in vitro and microbiological tests, and susceptibility to destruction by heating in the presence of a reducing sugar was investigated.

Covalent attachment of lysine ethyl ester or threonine to gluten by the plastein reaction utilizing the enzyme papain was not successful. Although lysine and threonine contents in the products were increased, these results were attributed to selective enzymatic release of other amino acids. The amino acid compositions of undialyzable "plastein" products were markedly different from the original gluten substrate, and product yields were low. The results suggest the formation of many dialyzable peptides and free amino acids, and inability for protein re-synthesis from these low molecular weight compounds.

Covalent lysine and threonine contents were increased using the carbodiimide reaction. In general, the reaction was most influenced by pH, reactant concentration and type of reactant. Products enriched via primarily peptide bonds as well as products enriched via peptide and isopeptide bonds could be prepared using various starting materials. Lysine, N^ε-acetyl lysine, N^ε-benzylidene lysine and threonine were coupled through amide bond formation to gluten, sodium stearate-solubilized gluten, acid-solubilized gluten or pepsin-solubilized gluten.

Sodium stearate solubilization did not improve extent of amino acid incorporation. Pepsin hydrolysis of gluten enhanced amino acid attachment but decreased product yields. 4.0-fold and 6.5-fold increases in lysine content resulted by reaction of pepsin-solubilized gluten with N^ε-benzylidene lysine and N^ε-acetyl lysine, respectively. However, yields of these products were low (47% and 58%). 1.6-fold, 2.0-fold and 2.5-fold increases were obtained by reaction of gluten with N^ε-benzylidene lysine, lysine and N^ε-acetyl lysine, respectively, with product yields of 90 to 95%. At least 20-fold and 5-fold increases could be achieved by reaction of 0.5N HCl and 0.05N HCl solubilized glutens respectively with lysine, with product yields of 80 to 90%.

4-fold and 2-fold increases in threonine content resulted from reaction of threonine with pepsin-solubilized gluten and gluten, respectively. Simultaneous attachment of lysine or lysine derivative and threonine was not effective.

In vitro evaluation of availability and digestibility of covalently enriched products was carried out by the DNBS reaction and pepsin pancreatin digestion tests. The results indicate the formation of isopeptide

bonds involving the ϵ -amino group of lysine unless N^ϵ -substituents of lysine were used. Isopeptide bonds involving the γ -carboxyl groups were indicated when gluten had been solubilized by acid treatment. Peptide bond formation predominated when N^ϵ -benzylidene or N^ϵ -acetyl lysine was attached to pepsin-solubilized gluten or gluten. The high in vitro availability and digestibility values for N^ϵ -benzylidene lysine enriched products suggest lability of the Schiff's base linkage of this N^ϵ -substituent, in contrast to the stability of the amide linkage in N^ϵ -acetyl lysine. Microbiological evaluation by a Tetrahymena bioassay confirmed the nutritional improvement of gluten by covalent attachment of lysine, N^ϵ -acetyl lysine or N^ϵ -benzylidene lysine. Relative nutritive value of gluten was 54, whereas covalently and freely enriched glutens had relative nutritive values similar to that of the reference casein, assigned a value of 100.

Covalently and freely lysine enriched glutens were compared for color and extent of lysine destruction after baking. In general, covalently enriched products had lighter color and higher percentages of total, DNBS-available and pepsin-pancreatin-digestible lysine contents than freely enriched products. N^ϵ -Benzylidene lysine enriched gluten was particularly stable, with relative nutritive value of 88 compared to 44 for baked gluten. It is concluded that covalently attached lysine is more stable than free lysine for enrichment of food proteins susceptible to Maillard reaction.

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GLOSSARY OF SYMBOLS

lys.HCl = lysine monohydrochloride

AL or N^εAcLys = N^ε-acetyl lysine

BL or N^εBenz.Lys. = N^ε-benzylidene lysine

x% lysine-gluten = gluten covalently enriched with x% lysine (w/w
ratio of lysine equivalent to gluten)

gluten + x% lysine = gluten enriched by addition of x% lysine (w/w
ratio of lysine equivalent to gluten)

$\alpha-\alpha$, $\gamma-\alpha$, $\alpha-\epsilon$, $\gamma-\epsilon$ = designation of amide bonds formed
by the condensation of α - or γ -carboxyl groups
with α - or ϵ -amino groups ($-\overset{\text{O}}{\underset{\text{||}}{\text{C}}}-\text{NH}-$)

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INTRODUCTION

The fulfilment of protein, energy and other nutritional requirements of population groups is largely governed by agricultural and economic resources available in the respective geographic areas. Preference for animal protein sources appears to increase with the affluence of the society (Wilcke and Altschul, 1978). However, the future of animal protein depends on the crucial question of the continuing adequacy of total food supply throughout the world. More edible protein is produced per unit of land area by plant crops than by animal production (Christensen, 1948), and it was to this fact that criticism was aimed during the World Food Conference in Rome in 1974 (Wilcke and Altschul, 1978). An ever-present controversy questions the morality of feeding grain to animals to satisfy the desires of a few, at the expense of using this grain to alleviate the hunger of many others. Brown (1974) calculated the per capita availability of grain to be almost one ton per year per individual in North America. However, of this, only 200 lb. are consumed directly, while the remainder, 1700 to 1800 lb., is consumed indirectly after production of meat, milk and eggs.

Two possibilities for increasing protein supply to meet world-wide demand are firstly, to directly increase the utilization of grain and vegetable proteins for human consumption while decreasing animal protein consumption, or secondly, to improve the efficiency of converting grain proteins to animal proteins for human consumption. In either case, the protein impact of grain sources is limited by their quality, for the efficient use of any protein by both man and animals is dependent on the

proper balance and levels of essential and non-essential amino acids to meet specific physiological requirements.

Proteins derived from vegetable origin are virtually all deficient in one or more of the essential amino acids (Bressani, 1975). For example, cereal grains are deficient in lysine, with corn and rice having further deficiencies in tryptophan and threonine, respectively. (Jansen, 1974). Protein quality can be improved by addition of the limiting amino acids, and cereals are especially prime candidates for amino acid fortification because they generally would provide adequate supplies of dietary protein to meet the needs of even young children, if only the protein quality was improved. For example, the universal acceptability of wheat, its production in vast quantities, its high protein content and excellent digestibility have all promoted programs to facilitate fortification and use of wheat to counter-attack the effects of malnutrition throughout the world (Graham et al., 1969). As early as 1914, it was demonstrated that wheat protein could be improved nutritionally by addition of lysine (Osborne and Mendel, 1914). Currently, the Agency for International Development (AID) is sponsoring field trials on the fortification of wheat with lysine in Tunisia (Altschul, 1974).

While simple addition of the limiting amino acids in free form is a convenient means for protein fortification which is currently in practice, particularly in animal feed formulations, this is not necessarily the best approach for protein quality improvement. Added amino acids may be easily lost during food processing steps such as washing or discarding of cooking water (Fujimaki et al., 1977). The free amino acids

may be more susceptible than protein-bound amino acids to destructive chemical reactions such as the Maillard browning reaction, or to attack by microbial enzymes during storage (Eskin et al., 1971).

Undesirable flavor and odor properties may be imparted by free amino acids such as methionine (Klaui, 1974; Hippe and Warthesen, 1978).

The efficient utilization of the added amino acids in vivo may decrease if their rates of digestion and absorption are different from amino acids derived from intact protein or peptides (Rolls et al., 1972). Graham et al. (1969) suggested less effective utilization of L-lysine monohydrochloride added to wheat flour than protein-bound lysine when fed to infants, possibly due to faster absorption than the amino acids derived from digestion of wheat protein. Differences in transport have been reported between oligopeptide methionine and free methionine (Lis et al., 1972) and between oligopeptide lysine and free lysine (Burston et al., 1972).

Covalent attachment of limiting amino acids may circumvent the above-mentioned problems of free amino acid fortification. If so, it may be the preferred means for improvement of protein quality in terms of both nutritional and functional properties. The specific objectives of this study were (1) to investigate the enzymatic plastein reaction and the chemical carbodiimide reaction as means for covalent attachment of lysine and threonine to wheat gluten or solubilized wheat gluten; (2) to evaluate the nutritional quality of these covalently enriched glutes by (a) in vitro chemical and enzymatic tests and (b) in vivo microbiological assay; and (3) to determine the relative susceptibility of the covalently

lysine enriched gluten to browning during model baking studies, compared to susceptibility of corresponding samples enriched by free addition of lysine.

LITERATURE REVIEW

A. WHEAT GLUTEN

I. Solubility properties and solubilization techniques

When wheat flour is wetted with water and mixed, a complex cohesive and elastic mass is formed. Wheat gluten, which makes up 80 – 85% of the wheat flour proteins, is primarily responsible for the framework of wheat flour dough and for the integrity which permits gas retention for production of baked products such as bread.

Gluten is composed of two major protein fractions, an alcohol-soluble fraction termed gliadin and an acidsoluble fraction termed glutenin (Krull and Wall, 1969). Gliadin is cohesive, while glutenin is both cohesive and elastic (Dimler, 1963), and these proteins impart in essence the unique dough-forming properties of wheat flour.

The amino acid compositions of gluten and the fractions gliadin and glutenin are shown in Table 1. Although some differences exist between the two fractions, gluten proteins are generally characterized by unusually high glutamine and proline contents, low ionizable amino acid contents (eg. lysine, histidine, arginine, aspartic and glutamic acids) and high proportions of non-polar amino acids (eg. valine, leucine, isoleucine). These observations suggest the likelihood of a predominance of hydrogen bonding and hydrophobic associations rather than electrostatic interactions, and may explain the solubility characteristics of gluten (Krull and Wall, 1969). Wheat gluten is insoluble in water, but may be dissolved in aqueous urea solutions or in acidified aqueous solutions with low ionic strength.

Table 1. Amino acid composition of gluten, gliadin and glutenin
(adapted from work cited by Kasard, et al., 1971)

amino acid	moles amino acid/ 10^5 g. protein		
	gluten	gliadin	glutenin
arg	20	15	20
his	15	15	13
lys	9	5	13
thr	21	18	26
ser	40	38	50
asp	22	20	23
glu	290	317	278
gly	47	25	78
ala	30	25	34
val	45	43	41
leu	59	62	57
ile	33	37	28
pro	137	148	114
tyr	20	16	25
phe	32	38	27
trp	6	5	8
cys/2	14	10	10
met	12	12	12
NH ₃	298	301	240

Various workers have attempted to improve the solubility of gluten in aqueous solution. Holme and Briggs (1959) used selective mild acid hydrolysis with 0.008 to 0.04N HCl to convert glutamine to glutamate residues, with resultant increase in protein solubility. Grant (1973) modified wheat flour with succinic anhydride and improved solubility. Wu et al. (1976) compared hydrochloric and acetic acids for solubilization of gluten, and concluded that solubilization could be achieved when the amide content was reduced by approximately 10%. Fung et al. (1977) similarly prepared acid-solubilized flour by simultaneous solubilization of protein and starch with hydrochloric acid. Kobrehel and Bushuk (1977) studied the effects of long chain fatty acids and their sodium salts on solubility of glutenin in water and showed significant improvements in solubility by the addition of relatively high concentrations of sodium palmitate or sodium stearate.

Controlled proteolysis has been used for fractionation of gluten and may be applied for production of solubilized gluten. Yang and McCalla (1968) concluded that trypsin was considerably less effective for hydrolysis than either papain or pepsin; papain produced more non-protein nitrogen most rapidly during early stages of hydrolysis, although pepsin produced the largest total after 48 hours. Bleitz and Rothfus (1970) used pepsin for fractionation of gliadin and glutenin into peptides, while Finlayson (1964) utilized pepsin followed by trypsin for gliadin hydrolysis. Oka et al. (1965), in a study on the action of pepsin on glutenin, observed the rapid decrease in the viscosity of glutenin dispersions, accompanied by increase in solubility at neutral pH, and attributed this to selective pepsin cleavage of relatively few peptide bonds

to produce comparatively large yet water-soluble polypeptides. Further pepsin action produced substantially smaller peptide fragments. Draudt et al. (1965) used pepsin to increase solubility of wheat protein in efforts to prepare milk-like products.

2. Nutritional quality and improvement by fortification

The composition of essential amino acids in wheat gluten is compared to that of a recommended reference pattern in Table 2. Lysine is the first limiting amino acid, while threonine is the second limiting amino acid in wheat gluten. Based on these values, the chemical score for gluten is 26.1, compared to 100 for whole egg. On the basis of the second most limiting amino acid ; threonine, the chemical score would be 63.6 (Hackler, 1977).

The amino acid fortification of cereals for nutritional improvement has been extensively reviewed by Jansen (1974), who concluded that evidence for amino acid fortification is abundant and "demonstrates conclusively that the protein quality of all cereals can be improved considerably, as determined by rat growth methods, by the addition of one or two essential amino acids." As early as 1914, Osborne and Mendel (1914) demonstrated the improvement of wheat gliadin for rat growth by addition of lysine. Howe et al. (1965) reported protein efficiency ratio (PER) values of 0.65 for white flour, 1.56 for flour + 0.2% L-lysine monohydrochloride and 2.67 for flour + 0.4% L-lysine monohydrochloride + 0.3% DL-threonine, compared to a value of 2.50 for casein. Rosenberg and Rohdenburg(1952) studied fortification of white bread and

Table 2. Comparison of the essential amino acids for recommended reference pattern and composition of wheat gluten

	g/100 g protein	
	recommended reference pattern, (FAO, 1973)	gluten
isoleucine	4.0	3.2
leucine	7.0	6.5
lysine	5.5	1.3
methionine + cystine	3.5	2.8
phenylalanine + tyrosine	6.0	7.9
threonine	4.0	2.2
tryptophan	1.0	0.8
valine	5.0	3.0

obtained PER values of 1.01, 1.89 and 2.09 for unsupplemented, 0.25% and 0.50% L-lysine monohydrochloride supplemented breads, respectively. Bender (1958) and Rosenberg et al. (1960) showed that both lysine and threonine supplementation are required for maximal growth in rat. Samonds and Hegsted (1973) reported increases of relative nutritive value (RNV) of gluten for growth of young cebus monkeys from 15 to 48%, and for maintenance from 23 to 69%, when lysine was added to wheat gluten.

In humans, the significant improvement in protein quality of gluten or wheat products particularly for growth requirements has been demonstrated on studies with infants and young children. Albanese et al. (Albanese et al., 1956; Albanese, 1959) showed poor nitrogen retention for infants on gluten alone, but after 3 weeks, nitrogen retention values improved when gluten was supplemented with lysine so that the values were comparable to those from children fed on milk. Bressani et al. (1960) studied the effects of supplementing a wheat basal diet with limiting amino acids to meet FAO reference protein standards. Comparison of the essential amino acid pattern of the wheat diet with the reference protein showed the order of limiting amino acids for children as lysine, tryptophan, methionine, isoleucine, valine and threonine. Their study indicated that lysine supplementation alone produced sustained nitrogen retention similar to that obtained with milk or with complete supplementation of all the limiting amino acids. When the basal diet was supplemented with all the limiting amino acids except lysine, vomiting and reduction in nitrogen retention resulted. Barness et al.

(1961) found similarly that the protein value of wheat protein (in the form of commercial cream of wheat) supplemented with lysine and potassium was close to the value of milk, and the supplemented diet was an adequate source of protein for the infants. Graham et al. (1971) concluded on the basis of several studies that where wheat is the main source of protein for infants and young children, enrichment with 0.2% L-lysine monohydrochloride is recommended.

B. COVALENT ATTACHMENT FOR FORTIFICATION

1. Enzymatic reaction ("plastein" formation)

Proteolytic enzymes are generally associated with catalysis of peptide bond hydrolysis, giving rise to products with lower molecular weight and higher water solubility than the original substrate. However, if highly concentrated solutions of protein or peptide hydrolysates are incubated with proteases under appropriate conditions, water-insoluble and/or gel-forming products may be formed. The term "plastein" was first used by Sawjalow (1901) to describe these resynthesis products.

In order for the plastein reaction to proceed efficiently, the reaction conditions must be rigidly controlled. Among the most important parameters to consider are substrate concentration, average molecular weight of the substrate and pH of the reacting medium (Eriksen and Fagerson, 1976; Fujimaki et al., 1977; Borsook, 1953). Substrate concentration should be in the range of 20 – 40% (w/v) (Fujimaki et al., 1977), since at lower substrate concentrations, the tendency is for hydrolysis rather than synthesis (Wasteneys and Borsook, 1930). The

substrate must be of low molecular weight and it has been reported that a peptide size of four to six amino acid units is most favorable (Virtanen, 1951; Determann et al., 1963). The optimal pH for most proteases generally lies in the range of 4 to 7 for resynthesis, although the optimal pH for hydrolysis may vary from 1.6 for hog pepsin to 10 - 11 for Biopraxe, a serine protease from B. subtilis (Yamashita et al., 1971a). Based on the difference between the optimum pH values for synthesis and hydrolysis (Δ pH), Yamashita et al. (1971a) classified enzymes into three types: pepsin type (Δ pH > 0), chymotrypsin type (Δ pH < 0) and papain type (Δ pH = 0), with the exception of trypsin (not plastein-productive at any pH). Among the well-known proteinases, comparatively high plastein yields were obtained using pepsin, α -chymotrypsin and papain.

The plastein reaction has been investigated for many different applications in the food area. It may be used for the removal of unwanted compounds such as odorants (Arai et al., 1970; Fujimaki et al., 1968; Fujimaki et al., 1970a). Debittering of protein hydrolysates is effective through the plastein reaction (Arai et al., 1970; Fujimaki et al., 1970b). The plastein reaction has also been reported for preparation of products of high nutritional quality by incorporating controlled amounts of essential amino acids during the incubation. The plastein reaction was proposed for use of fish protein concentrate (FPC) and soybean protein isolate (SPI) to prepare a peptide-type low phenylalanine, high tyrosine food for curing phenylketonuria, with product yields of 69.3% from FPC and 60.9% from SPI (Yamashita et al., 1976). The nutritional quality of soybean protein was improved by incubating soybean protein hydrolysate

with L-methionine ethyl ester and papain under conditions favoring plastein formation; a plastein containing 7.22% methionine (compared to 1.18% methionine in the original protein) was obtained in 70% yield (Yamashita et al., 1971b; Arai et al., 1975a): Lysine content was increased by incubation of gluten which had been hydrolyzed by fungal alkaline protease (85% hydrolysis) with L-lysine ethyl ester and papain under the following conditions: 10 g. gluten hydrolysate, 5.0g L-lysine ethyl ester, 35% (w/v) substrate concentration, 20% acetone with 10mM cysteine(pH 6) reacting medium, 1:100 (w/w) enzyme:substrate ratio, 37°C, 48 hours. After incubation, ultrafiltration yielded a plastein with molecular weight > 500 and lysine content of 16% (Fujimaki et al., 1977).

Although numerous workers have been able to form water-insoluble products through the so-called plastein reaction for resynthesis of peptide bonds, the actual nature of the products and the mechanism for their formation is highly controversial. Peptide bond resynthesis could be occurring either by condensation or by transpeptidation. In the case of condensation, a decrease in free terminal amino groups should occur. Some workers have reported such a decrease (Wasteneys and Borsook, 1924) while others have not been able to detect it (Horowitz and Haurowitz, 1959). At the same time, an increase in the average molecular weight of the peptides should accompany condensation. Some workers have reported an increase in the molecular weights of plastein products (Yamashita et al., 1974; Tauber, 1951), but the validity of these results was considered questionable by v. Hofsten and Lalasidis (1976) due to poor solubility of plastein in water and ordinary buffers.

Using Sephadex chromatography (v. Hofsten and Lalasidis, 1976) and sodium dodecyl sulfate electrophoresis (Edwards and Shipe, 1978), no high molecular weight, protein-like material was obtained through plastein formation.

The other mechanism for peptide bond resynthesis is transpeptidation, which involves an interior peptide bond as a reactant. In this case, there may not necessarily be a decrease in free terminal amino groups, and whether or not there is an increase in the molecular weight depends on the relative sizes of the original peptides involved in transpeptidation. Horowitz and Haurowitz (1959) concluded that transpeptidation is the predominating mechanism in plastein formation by chymotrypsin, yet transpeptidation is hard to accept as the sole factor responsible for the formation of insoluble or gel-forming products (Eriksen and Fagerson, 1976).

In lieu of the hypothesis of plastein formation through resynthesis of covalent peptide bonds, some workers have suggested that hydrophobic bonding between peptides is the major force leading to water-insolubility, precipitation or gel formation of plasteins (Aso et al., 1973; Eriksen and Fagerson, 1976; v. Hofsten and Lalasidis, 1976; Edwards and Shipe, 1978). An increase in the proportion of nonpolar or hydrophobic amino acids in the water-insoluble reaction product in comparison to the original reactant has been reported (Aso et al., 1974) and the ratio of hydrophilic to hydrophobic peptides in the incubation mixture for the plastein formation definitely affects the properties of the plasteins (Arai et al., 1975b).

The term "plastein" thus should be used with caution to describe the enzymatic formation of water-insoluble products from protein or peptide hydrolysates (v. Hofsten and Lalasidis, 1976). Like "peptone", "plastein" may simply be a complex mixture of peptides, the former being a soluble mixture and the latter an insoluble mixture. The insolubility of plastein products may be due to the association of relatively small molecules through noncovalent interactions.

2. Chemical reactions

(a) Overview of application to food proteins

Chemical modification of proteins has been widely used in basic areas of protein research (Means and Feeney, 1971). However, the intentional chemical modification of food proteins is currently very limited, due to cultural and legal considerations as well as the potential health hazard. The literature cites many reports on the chemical modification of food proteins, yet the majority of these are on fundamental studies of the proteins from food rather than on the actual application of modification to solve practical needs (Feeney, 1977a; Feeney, 1977b).

Chemical modification offers great potential in improving and extending use of food proteins from both conventional and novel sources. Feeney (1977a, b) lists some of the possible applications. Chemical modification of food protein side chains may be used to improve nutritional quality by covalent binding of important nutrients to proteins or by increasing protein digestibility. It may prevent deteriorative reactions such as the Maillard reaction or control production of undesirable compounds such as lysinoalanine on exposure to alkali. It may improve

physical properties such as solubility or improve functional properties such as whippability.

Blocking of amino acid functional groups for the purpose of protection from deteriorative reactions has been carried out by the use of dimethylation (Galembeck et al., 1977), formylation, acetylation and propionylation (Bjarnason and Carpenter, 1970; Carpenter, 1973). Various acylating and alkylating reagents such as formaldehyde, N-methyloylacrylamide, hydroxyethylacrylate and N-vinyl-2-pyrrolidine were evaluated for their ability to modify casein to decrease its solubility and microbial degradation in the rumen (Friedman and Broderick, 1977). 3,3-Dimethylglutaric anhydride was used to modify egg white protein; foam formation was not seriously affected but heat coagulation properties were changed and a protective action against heat-induced changes in viscosity, light transmission and aerating ability was observed (Gandhi et al., 1968). Succinic and acetic anhydride have been used frequently to improve solubility and functional properties. Grant (1973) succinylated wheat flour proteins with the result of converting 95% of the protein to derivatives soluble in water. Groninger and Miller (1975), Miller and Groninger (1976) and Chen et al. (1975) studied the properties of acetylated and succinylated fish proteins, and reported rapid rehydration, good dispersion characteristics at neutral pH, and increasing emulsifying activity and capacity, gelation, water sorption, aeration and foam stability with increasing degree of acylation. Thompson and Reyes (1980) succinylated heat coagulated whey protein concentrates and suggested possible application of the product in situations requiring high water and fat absorption capacities, viscosity and emulsifying properties, such as

baked goods, processed meats and dairy analogues. McElwain et al. (1975) studied the effect of succinylation on properties of single cell protein concentrate and observed increase in emulsion viscosity but decrease in emulsion stability. Childs and Park (1976) acylated glandless cottonseed flour with succinic and acetic anhydrides and observed improved oil-holding and water-holding capacities with both reagents; succinylation increased emulsifying capacity while acetylation increased foam capacity.

To date, relatively little work has been published on the use of chemical modification for specific improvement of nutritional quality. Covalent introduction of phenylalanine, tyrosine, methionine and isoleucine into whey protein was reported in a note by Bjarnason-Baumann et al. (1977), using N-carboxyanhydride by stepwise condensation on an arginylpolyethyleneimine support as described by Pfaender et al. (1976). The relative nutritive values by the slope ratio assay with young rats were 0.75 for whey protein and 0.92 for the fortified whey protein (Bjarnasson-Baumann et al., 1977).

Casein was covalently modified via the ϵ -amino group of lysine residues using a series of active N-hydroxysuccinimide esters of tert-butyloxycarbonyl amino acids (Puigserver et al., 1979). N-hydroxysuccinimide esters have been used for peptide synthesis in aqueous solution (Anderson et al., 1963; Anderson et al., 1964). In vitro digestibility rate studies using bovine chymotrypsin, bovine pancreatin and rat bile pancreatic juice showed lower rates of hydrolysis of the modified casein derivatives compared to the unmodified protein. On the other hand, plasma amino acid patterns for rats fed a 10% protein diet of highly modified glycyl- or methionyl-caseins suggested good

in vivo digestibility (Puigserver et al., 1978a). However, one disadvantage of this method was the requirement for anhydrous trifluoroacetic acid for removal of the tert-butyloxycarbonyl protecting group. As Puigserver et al. (1978b) noted, although biological activity of several enzymes were found to be restored after trifluoroacetic acid treatment when returned to aqueous solutions, this treatment cannot be applied to proteins in foods because of the toxicity of trifluoroacetic acid.

Voutsinas and Nakai (1979) used a water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, for covalent binding of methionine and tryptophan to soybean protein. Fractional factorial experimental designs were used to investigate reaction conditions favoring the binding reaction. Increases in covalent methionine and tryptophan contents of 7.7-fold and 18.0-fold, respectively, were obtained using soy protein hydrolysate, with 16 ± 30% protein recovery. When soy protein isolate was used instead of soy protein hydrolysate, covalent methionine and tryptophan contents were increased 6.3-fold and 11.3-fold, respectively, with 95 - 99% protein recovery. An in vitro pepsin-pancreatin digestion test demonstrated that the bound amino acids were readily released. Gel filtration chromatography indicated increase in molecular weights of soy protein fractions after the carbodiimide reaction but no selective amino acid binding was observed in the different soy protein fractions.

Holbek (1976) and Chau (1977) used the carbodiimide method for binding of lysine to 0.5N HCl acid-solubilized gluten. Using fractional factorial experiments (Taguchi, 1957), Holbek (1976) and Chau (1977) showed that pH ($P < 0.01$), temperature ($P < 0.05$) and lysine

amount ($P < 0.05$) were significant parameters influencing the extent of binding of lysine during the carbodiimide reaction. In these experiments, up to 20-fold increase in covalent lysine content could be achieved. Trifluoroacetyl, formyl, benzylidene and tert-butyloxy-carboxyl groups were studied as possible protecting groups for the ϵ -amino group of lysine to promote α - α peptide bond formation during the carbodiimide reaction. Of these, only the N^ϵ -benzylidene lysine was incorporated; no significant lysine increase was observed when any of the other three derivatives were used (Holbek, 1977).

(b) Carbodiimide reaction

The formation of an amide or so-called peptide bond in peptide and protein molecules involves the acylation of the amino group of an amino acid by the carboxyl group of another amino acid. This reaction requires the input of energy, usually in the form of activation of the carboxyl group (Bodanszky et al., 1976). Carbodiimides are very useful activators of carboxyl groups and dicyclohexylcarbodiimide, first proposed for this purpose by Sheehan and Hess (1955), has been described as perhaps the most useful and popular coupling reagent (Bodanszky et al., 1976). Addy et al. (1973b) suggested that carbodiimides may have existed in primordial times as isomers of protonated cyanamide anion, and that through the carbodiimides, selective incorporation of amino acids would have led to peptides and proteins with primitive biological functions.

The popularity of carbodiimides in the synthesis of peptides is probably attributable to a number of factors. It is very reactive and

gives high yields under mild conditions within short periods of time (Bodanszky and Ondetti, 1966). It is commercially available. Unlike many other reagents used in peptide synthesis, carbodiimides do not require anhydrous conditions and the reaction can therefore be carried out in aqueous media (Kurzer and Douraghi-Zadeh, 1967). The possibility of racemization depends on the reaction conditions, being minimized by low temperature and nonpolar solvents (Anderson and Callahan, 1958; Bodanszky et al., 1976). Rebek and Feitler (1975) reported negligible extents of racemization. The addition of N-hydroxysuccinimide or 1-hydroxysuccinimide during the carbodiimide reaction has been reported to be efficient in reducing racemization (Weygand et al., 1966; Jones, 1974; Bodanszky et al., 1976). Toxicity and antitumor properties of some carbodiimides have been examined; di(triphenylmethyl) carbodiimide was much more toxic to malignant than normal cell lines while dicyclohexylcarbodiimide showed antitumor toxicity (Kurzer and Douraghi-Zadeh, 1967). The mammalian toxicity of carbodiimides is low, the LD₅₀ of dicyclohexylcarbodiimide being 2.6 g/kg in rats (Aumuller, 1963).

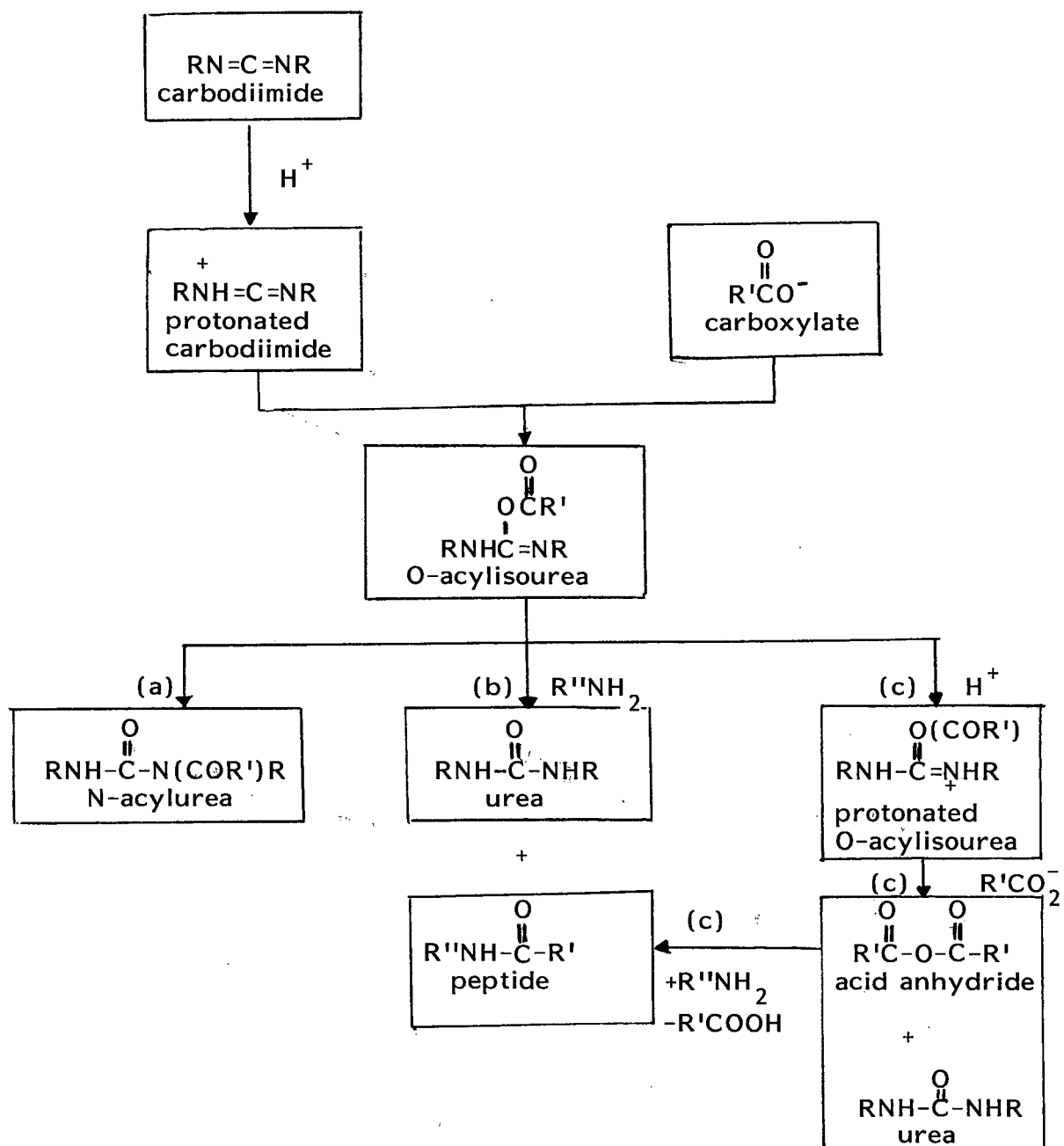
Use of the carbodiimides which were originally proposed for peptide synthesis, including N,N'-dicyclohexylcarbodiimide, is often plagued by problems of purification of the end products. These carbodiimides as well as the N-acylurea by-product and N,N-disubstituted isourea co-product all have poor solubility, a characteristic shared by the desired end product, the peptide (Bodanszky et al., 1976). The advent of water-soluble carbodiimides such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide solved this problem as the carbodiimide and its urea and acylurea derivatives are generally soluble in water, and thus

can be easily removed from the desired product by washing or dialysis using dilute acid or water. Use of these water-soluble carbodiimides has permitted synthesis of peptides under mild aqueous conditions in high yields and pure form (Sheehan and Hlavka, 1956; Sheehan et al., 1965; Greenstein and Winitz, 1961).

The mechanism of amide formation under the influence of carbodiimides was first explored by Khorana (1953, 1955) and has since been re-explored by many other workers, including DeTar et al. (1966a, b, c). Initial protonation of the carbodiimide is required to yield the intermediate which is then attacked by the carboxylate anion to produce the O-acylisourea. The O-acylisourea is itself an unstable reactive intermediate which can in turn react by three pathways, as shown in Figure 1 (adapted from Kurzer and Douraghi-Zadeh, 1967). It may form the N-acylurea by intramolecular rearrangement (pathway (a)); this is favored by the absence or poor reactivity of nucleophiles in the reacting medium and by lack of protons (Perfetti et al., 1976). In the presence of nucleophile (eg. an amine $R''NH_2$), the O-acylisourea can either form the peptide and urea co-product directly (pathway (b)), or if excess carboxyl groups are present, form the peptide and urea co-product via incorporation of a second carboxylate group to form the acid anhydride, which in turn reacts with the nucleophile (pathway (c)). Delayed addition of the nucleophile favors pathway (c) over pathway (b) for synthesis of the peptide (Rebek and Feitler, 1973).

The carbodiimide method has been investigated for quantitative estimation (Hoare and Koshland, 1966; Hoare and Koshland, 1967) and selective modification (Perfetti et al., 1976) of carboxyl groups in proteins.

Figure 1. Scheme for amide ("peptide") bond formation via the carbodiimide reaction.



The accumulation of N-acylurea which would preclude amide bond formation may be discouraged by addition of a strong nucleophile such as glycine methyl ester (Hoare and Koshland, 1967).

The pH of the reaction is critical and is generally about 4.5 for modification of carboxyl groups in proteins (Means and Feeney, 1971). Since the reaction requires the protonated form of the carbodiimide and the unprotonated forms of the carboxyl and amino groups, there is a strong dependence of peptide synthesis yield on acid concentration (Addy et al., 1973a). Acid concentration is also critical for stability of the carbodiimide. Carbodiimides react additively with water to form ureas, this reaction being catalyzed both by acids and alkalis (Kurzer and Douraghi-Zadeh, 1967). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide has been reported to be relatively stable for long periods of time (Hoare and Koshland, 1967).

Since proteins may have side chain β - and γ -carboxyl groups from aspartyl and glutamyl residues respectively, it is possible to activate these groups in addition to activation of the α -carboxyl terminal group. To have selective carboxyl terminal activation for nucleophile attack, it is necessary to have complete absence of all nucleophiles from the activation (pre-incubation) mixture during the incubation (Previero et al., 1973). The extent of nucleophile incorporation by aspartyl or glutamyl peptides was shown to be doubled by direct condensation, compared to condensation after suitable pre-incubation of peptide with carbodiimide only (Previero et al., 1973).

Yield of incorporation of a nucleophile such as an amine to form a peptide bond depends also on the nature of the nucleophile. Addy et

al. (1973b) showed a general increase in yield with size of the aliphatic side chain of amino acids. The microenvironment at the reaction center was postulated to contribute to the selectivity for certain amino acids for the peptide synthesis reaction.

Intramolecular as well as intermolecular crosslinks may be formed by the carbodiimide reaction since amide bond formation may occur between activated carboxyl groups and amino groups on the same or another protein molecule. Atassi and Singhal (1972) concluded that intermolecular crosslinks could be greatly minimized by carrying out the reaction on a relatively dilute mixture of reactants. Timkovich (1977) reported that nucleophilic addition reactions are less susceptible to intermolecular crosslinking or polymerization, but cautioned that polymerization cannot be assumed to occur only at elevated pH or in the absence of excess added nucleophile since this depends on the particular protein studied.

C. EVALUATION OF PROTEIN NUTRITIONAL QUALITY

Increasing awareness and concern regarding nutritional labelling requirements, nutrient destruction during processing and storage, and development of novel or improved food products have emphasized the urgency to develop rapid tests suitable for routine screening of proteins for nutritional value (Evancho et al., 1977; Landers, 1975). Although in the final analysis, the evaluation should be carried out on human subjects, the complexity of human assays precludes them from monitoring food development work, quality control or processing conditions, due to ethical and practical reasons. Fortunately, several reviews on the correla-

tion between human and animal protein quality assays have strongly suggested that protein quality assays in humans classify proteins in the same order as assays using experimental animals such as rats classify them (Bressani, 1977).

The official procedure currently in use in both Canada and the United States for the biological evaluation of protein quality is based on "Protein Efficiency Ratio" or "PER" determination (AOAC, 1975). This method employs 10% protein diets which are fed ad libitum to weanling rats for a 28 day period. Weight gain per unit of ingested protein is compared for test diets and control diets containing casein as the protein. The procedure is lengthy, tedious and expensive, and has numerous inherent problems (Evans and Witty, 1980; Jansen, 1978; Bodwell, 1977). PER values are dependent on the stage of growth of the test animals and on the conditions of the animals before the test is conducted. The weight gain is strongly dependent on food intake, the results being positively correlated with food intake (Hegsted and Chang, 1965). The method considers growth requirements and thus does not do justice to proteins which are adequate for maintenance but not for growth. The rat test has been described as a severe test for protein quality and may well underestimate the quality of many dietary proteins for adult man (Samonds and Hegsted, 1977). Difficulties also arise due to the adaptive response to low levels of limiting amino acids such as lysine. For example, Chu and Hegsted (1976) showed drastically reduced levels of the enzyme lysine ketoglutarate reductase in the liver of adult rats fed wheat gluten, which is first limiting in lysine. The reductase enzyme is presumed to be the first step in lysine degradation and decreased

enzyme levels may be an adaptive response for conservation of body lysine. In such cases, it becomes impossible to evaluate the true protein quality (Hegsted, 1977).

Improvements over the official PER procedure have been recommended. Tests which are based on net protein utilization, net protein ratio and biological value try to correct for the major flaw of the PER method (which is a single protein level method) by including a control group of animals fed a diet with no protein, to take into account loss of body weight or nitrogen in the latter case. Yet these methods are still of limited statistical validity, being two-point assays; the tests are also too time-consuming to perform as routine screening procedures (Samonds and Hegsted, 1977). The determination of relative nutritive value using the slope ratio assay and the determination of relative protein value using the modified slope ratio assay (Samonds and Hegsted, 1977) compare the slope of test protein to slope of a standard protein over ranges of protein intake and thus are better able to estimate a protein's ability to support growth in rats. However, even with these tests, the validity and reproducibility are dependent on rigid control over techniques in rat bioassays, including adaptation of laboratory rats, careful choice of strain, etc. (Miller and Lachance, 1977). The use of genetic potential curves for a control group of rats has been recommended to indicate involvement of an uncontrolled stressor in the experimental procedure, but these curves take time to develop (Miller and Lachance, 1977).

Clearly, protein quality evaluation with experimental animals can at best give rather limited information and with great expense of time and

money. For the purposes of screening and routine assays, procedures using microorganisms have been suggested. These procedures may only give as much information as animal feeding tests, but at least they do so with greater rapidity since the generation time of microorganisms is generally much shorter than animals. Reduction in quantity of test protein required for the assay, greater ease in handling many samples at a time and easy maintenance of culture for subsequent assays are all additional advantages.

Microbiological assays with bacteria have often been advocated for rapid screening tests, but most suffer from lack of specificity since many of the amino acid requirements can be overcome by other metabolites such as vitamins (Frank *et al.*, 1975). On the other hand, the protozoan Tetrahymena pyriformis W has a mammalian-like requirement for amino acids (Frank *et al.*, 1975). T. pyriformis was first proposed for use in protein quality determinations by Pilcher and Williams (1954) and since then, many studies have investigated its application for routine evaluation of protein quality (Evans and Witty, 1980).

The quantitative amino acid requirements of T. pyriformis have been investigated (Rølle, 1975; Evans, 1978). Table 3 compares these requirements with the FAO/WHO provisional scoring pattern (FAO/WHO, 1973) and the typical amino acid compositions of Animal Nutrition Research Council (ANRC) casein (approved as a reference protein for use in biological assay) and wheat gluten. With the exception of serine, which is required only by T. pyriformis, all amino acids essential for man and rat are needed for the growth of Tetrahymena (Frank *et al.*, 1975). The quantitative amino acid requirements of the organism are sufficiently

Table 3. Comparison of amino acid requirements of *T. pyriformis*, FAO/WHO provisional scoring pattern, and amino acid compositions of ANRC casein and wheat gluten.

g/100 g					
	<i>T. pyriformis</i> requirement ^a		FAO/WHO pattern ^b	ANRC casein ^c	wheat gluten ^d
arg	3.3	ND ^e	–	3.6	3.0
his	2.6	ND	–	2.5	1.8
ile	3.2	6.6	4.0	4.8	3.2
leu	8.8	6.6	7.0	8.8	6.5
lys	6.2	3.9	5.5	7.4	1.3
met + cys	2.9	2.7	3.5	2.5	2.8
phe + tyr	4.5	4.0	6.0	9.9	7.9
thr	6.7	6.6	4.0	3.9	2.2
trp	0.9	0.3	1.0	1.1	0.8
val	6.7	5.6	5.0	5.6	3.0
ser	5.7	ND	–	5.6	4.5

^a first column from Evans (1978); second column from Rølle (1975).

^b FAO/WHO (1973)

^c typical composition, from Humko Sheffield (1978)

^d Li-Chan and Nakai, unpublished results

^e "ND" indicates not determined.

similar to those of the human for growth to justify use of this organism in protein quality assessment (Evans and Witty, 1980). The amino acid composition of ANRC casein is generally similar to Tetrahymena requirements, thus indicating it is probably a good choice as a reference protein, while the low levels of lysine and threonine in wheat gluten suggest that it will be a poor protein source for supporting growth.

In order for a protein quality assay to be useful, protein must be the limiting factor in the test conditions. Many studies have been carried out to investigate the requirements of T. pyriformis in terms of nutritional and environmental factors affecting its growth, as has been reported in an excellent review by Evans and Witty (1980). Nucleic acid, mineral, vitamin, carbohydrate and fat requirements have been thoroughly studied, and most assay procedures currently in use are based on the original recommendations of Stott et al. (1963). In terms of amino acid requirements T. pyriformis can degrade intact proteins to release the amino acids required for growth, since it possesses enzyme systems somewhat comparable to those of higher animals (Kidder and Dewey, 1951). However, solubilization of protein is required for good correlation of T. pyriformis growth with animal growth indices or chemical indices (Landers, 1975; Evans and Witty, 1980), and various enzymatic pre-digestion procedures have been suggested for this purpose, including use of pepsin (Landers, 1975; Evancho et al., 1977), papain (Evans et al., 1978), trypsin and bromelain combination (Frank et al., 1975) and trypsin, chymotrypsin and peptidase combination (Dryden et al., 1977). However, although solubilization is recommended, sterile filtration is not advised since the presence of some particulate matter is required for inducing

formation of food vacuoles in the organism, thus promoting rapid multiplication of the cells (Rasmussen and Kludt, 1970).

In general, protein fractions need not be isolated from the test material and aliquots of the entire foodstuff are incorporated into the media to give a specified protein level (Evans and Witty, 1980). This may lead to biases if un-identified stimulatory or inhibitory substances are in the test material. High fat contents can depress growth by creating a barrier between the liquid media and the air needed for growth (Evans et al., 1978). Fat extraction with solvents is necessary in these foods, but removal of unknown components can affect growth. For example, whole egg defatted with diethyl ether supports a population 20 - 25% greater than egg defatted with alcohol, when these extracted fractions are used to provide protein at 1 - 2 mg N/ml (Rosen and Fernell, 1956).

Aside from nutritional factors, strict control is required over environmental factors such as temperature, incubation vessel, aeration, pH, salinity and light (Kidder and Dewey, 1951; Rosen and Fernell, 1956; Minato, 1980). Including the standard protein casein in each assay is recommended since variation from trial to trial on total count at a given nitrogen level was reported to be large even with a standard casein and strict control of the inoculant level; biological variation and unexpected fluctuations could not be avoided (Warren and Labuza, 1977).

In a comparison of the rat versus T. pyriformis assays, Evancho et al. (1977) reported the error inherent in both the rat PER bioassay and the Tetrahymena bioassay with direct microscopic count to be $\pm 10\%$, the error in the latter case being attributed largely to the use of direct

microscopic counting for growth measurement. Warren and Labuza (1977) reported a standard deviation for the control of $\pm 18.6\%$ relative nutritive value (RNV), based on values over a two year period. The standard deviation was attributed to variation in the casein to some extent, but it was felt again that problems in the counting procedure contributed most of the error.

The direct microscopic counting technique has been criticized but has been retained by the majority of workers as the primary manifestation of growth. The technique is tedious and time-consuming, and subject to error due to difficulty in obtaining a representative aliquot for counting; human error due to eye fatigue is a contributing factor as well. However, it does have some advantages. Since cells are placed in preserving fluid, they may be kept for long periods of time, and counted when convenient (Wang et al., 1979); contamination by other microorganisms can be easily visualized so that deviation from aseptic techniques may be easily monitored.

Use of a Coulter particle counter has been suggested to facilitate counting (Evancho et al., 1977; Teunisson, 1971). However, food particles are not distinguished from Tetrahymena cells when similar in size. In addition, Tetrahymena cell size varies with a number of conditions, including nutrient composition of medium, temperature of incubation, phase of growth, osmolarity of the medium, etc. (Hill, 1972). Media containing casein as the protein source support growth of cells which are large, turgid and oblong, whereas media containing bread crumb samples result in typically small and flat cells (Kaestner et al., 1976).

Wang et al. (1979) compared growth measurement using four

techniques and concluded that tetrazolium dye reduction, oxygen uptake or ATP-bioluminescence are all feasible alternatives to the direct microscopic counting technique. All techniques have inherent advantages and disadvantages. Although the dye reduction test is simple in technique and instrumentation, and measures only viable cells, it cannot differentiate between the desired organism and microbial contaminants; high concentrations of organisms (greater than 10^5 /ml) are required. The oxygen uptake test is theoretically straightforward, but requires technical expertise and expensive equipment and can only analyze small numbers of samples at a time. The ATP-bioluminescence method is simple and rapid, but Evancho et al. (1977) reported its lack of sensitivity.

Despite the limitations of the Tetrahymena bioassay, it is gaining popularity and has been applied successfully in various aspects of food protein evaluation. Wang et al. (1979) evaluated bread samples containing wheat flour, soybean meal or nonfat milk solids, and samples of diet mixtures containing ANRC casein, peanut meal or wheat gluten, and obtained good correlations between Tetrahymena relative nutritive values and rat PER values. In a study with commercially prepared frozen foods, Evancho et al. (1977) obtained a regression equation relating relative nutritive value by Tetrahymena (RNV) to PER ($PER = 0.286 + 0.022(RNV)$), with $r = 0.90$ ($P < 0.01$). Warren and Labuza (1977) studied the loss of overall protein quality due to nonenzymatic browning. The Tetrahymena bioassay was unable to detect the loss of available lysine as measured by a chemical test during the early stages of browning, but at advanced stages of browning, it showed equivalent or greater losses of nutritional value. Srinivas et al. (1975) used the bioassay to detect protein quality

changes in irradiated foods including wheat and shrimp and concluded negligible effects by irradiation. Lower cell counts using wheat proteins instead of casein as the protein source suggested good correlation of growth response to protein quality, and the improved growth response on addition of limiting amino acids to wheat flour indicated sensitivity to nutritional stress and specificity in essential amino acid requirements for optimum growth (Srinivas et al., 1975). Kaestner et al. (1976) used the bioassay to evaluate some crop cultivars and concluded that gross differences in essential amino acid content or availability could be detected, but extreme sensitivity of the organism to various growth conditions limits the method for distinguishing between samples with small amino acid composition differences. Kaestner et al. (1976) also concluded that after baking, the crusts of both wheat and rye bread had considerably reduced relative nutritive values, while the rye bread crumbs were superior to wheat crumbs.

In general, most workers conclude the feasibility of potential large scale application of the Tetrahymena bioassay as a screening test for protein quality, provided further studies to standardize and refine the procedure are achieved (Frank and Baker, 1975; Helms and Rølle, 1975; Landers, 1975; Kaestner et al., 1976; Evancho et al., 1977; Warren and Labuza, 1977; Hsu et al., 1978; Evans and Witty, 1980).

MATERIALS AND METHODS

A. Materials

Vital gluten was obtained commercially as "Whet Pro 75% vital wheat gluten" from Industrial Grain Products Ltd., Thunder Bay, Ont. L-Lysine monohydrochloride, L-lysine ethyl ester dihydrochloride (L-lys-OEt.diHCl, grade II) and L-threonine were obtained from Sigma Chemical Co., St. Louis, Mo. N^ε-Benzylidene lysine was synthesized by the method of Bezas and Zervas (1961); after the carbodiimide reaction was complete, intentional cleavage and removal of the protecting groups could be accomplished by heating in 1N HCl at 50°C for 15 min, followed by ether extraction. N^ε-Acetyl lysine was synthesized according to Leclerc and Benoiton (1967), using phenyl acetate from Matheson, Coleman and Bell. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS) and sodium stearate were all from Sigma Chemical Co. The proteolytic enzymes pepsin (from hog stomach mucosa, two times recrystallized), pancreatin (from porcine pancreas, grade VI) and papain (two times recrystallized) were from Sigma Chemical Co., while "Pronase" (grade B) was a product of Calbiochem. Animal Nutrition Research Council (ANRC) casein was a product of Humko Sheffield Chemical, Norwich, NY. Tetrahymena pyriformis W (ATCC) was purchased from the American Type Culture Collection, Rockville, Md.

B. Gluten solubilization

1. Pepsin hydrolysis

Partial pepsin hydrolysis was carried out by incubating dispersions

of gluten at pH 2 and 37°C with specified amounts of pepsin for varying time periods; the reaction was stopped by pH adjustment to neutrality, followed by freeze-drying to obtain powdered pepsin hydrolysates of gluten.

2. Acid hydrolysis

Mild acid hydrolysis (0.05N or 0.5N HCl, 121°C, 15 min) of gluten dispersions was carried out according to Wu et al. (1976). After hydrolysis, samples were neutralized and freeze-dried to obtain acid hydrolysates of gluten.

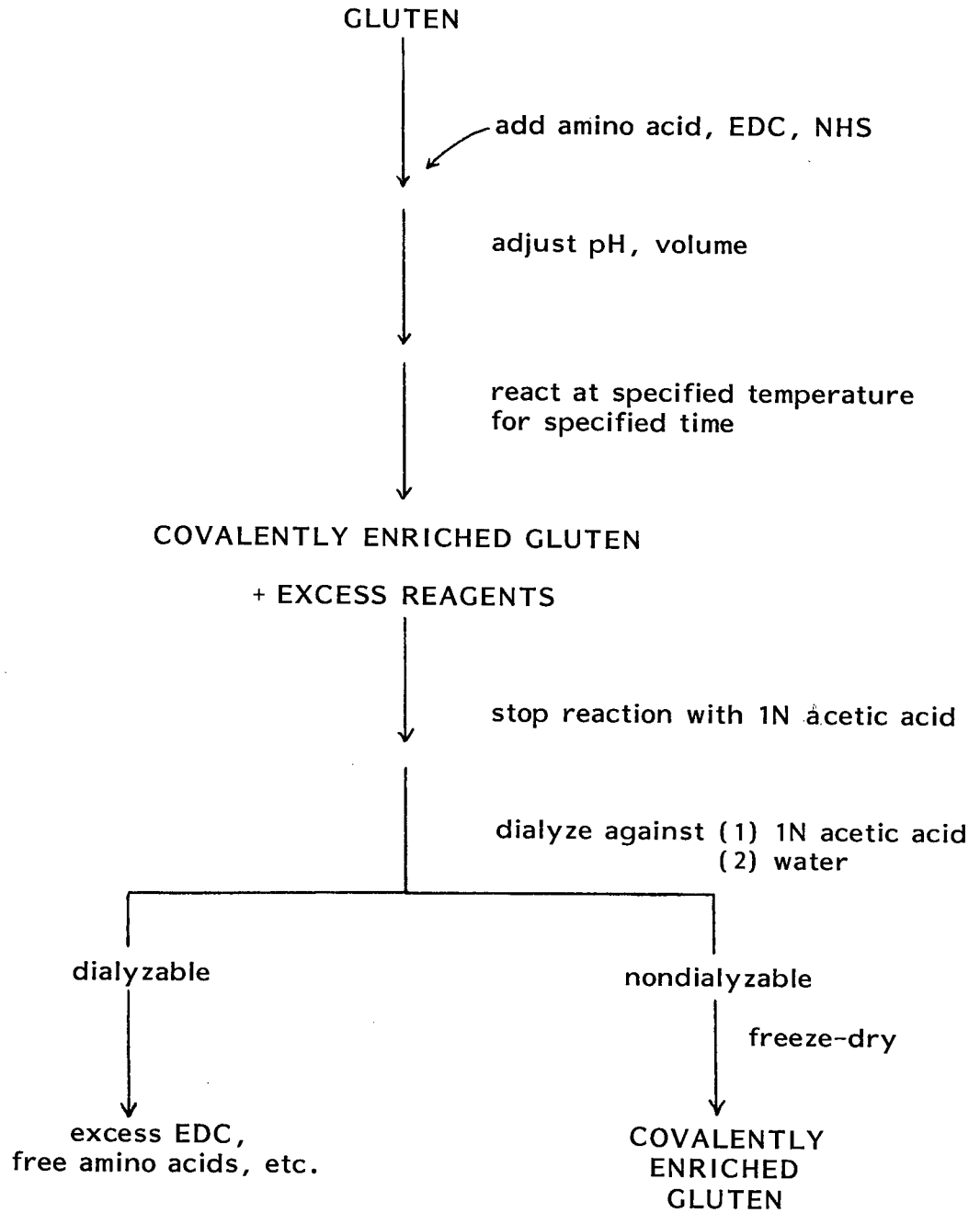
3. Sodium stearate addition

Solubilization of gluten by addition of sodium stearate was essentially according to the method of Kobrehel and Bushuk (1977). Gluten dispersions were gently shaken for 2 hours with 10% sodium stearate (w/w ratio of stearate to gluten) prior to the addition of reagents in the carbodiimide reaction.

C. Carbodiimide reaction for covalent attachment of amino acids to wheat gluten

Figure 2 illustrates the basic scheme for the carbodiimide reaction for covalent attachment of amino acids (lysine, N^ε-acetyl lysine, N^ε-benzylidene lysine or threonine, as specified) to gluten (acid- or pepsin- or sodium stearate-solubilized, or untreated gluten, as specified). Freshly prepared aqueous solutions of amino acid and EDC were added to a solution containing 500 mg gluten. NHS was added to reduce the possibilities of racemization and formation of N-acylureas (Weygand et al., 1966). Where EDC activation was indicated, EDC and gluten were

Figure 2. Carbodiimide reaction scheme for amino acid attachment to gluten.



pre-incubated prior to addition of amino acid. The pH and volume were adjusted. After incubation, the reaction was stopped by the addition of glacial acetic acid to 1N concentration, followed by dialysis at 4°C against two changes of 1N acetic acid over a period of 1½ days, then against running water for 1 day and finally against distilled, deionized water for another day. After pH adjustment to neutrality, the covalently enriched gluten (non-dialyzable portion) was freeze-dried. In preliminary experiments, dialysis against 1N acetic acid was succeeded by dialysis against 8M urea containing 0.2M mercaptoethanol, prior to dialysis against water. This step was included as a precautionary measure to avoid inclusion of non-covalently bound, physically entrapped free amino acids with covalently attached amino acids during determination of amino acid content of enriched samples. Since no significant difference in amino acid composition was observed whether or not urea-mercaptoethanol dialysis was carried out, this step was eliminated in subsequent experiments.

D. Plastein reaction to improve amino acid balance of wheat gluten

The plastein reaction described by Fujimaki et al. (1977) was attempted for improvement in the amino acid balance of wheat gluten. As substrates, either gluten, acid-solubilized gluten (0.05N HCl, 121°C, 15 min) or pepsin-hydrolyzed gluten (48 hour hydrolysis with 1% (w/w) pepsin, pH 2, 37°C) was used with lys-OEt.diHCl or threonine. Papain (1%, w/v) was incubated with the substrate mixture (35%, w/v) in the presence of 0.01M cysteine, 20% acetone at pH 6.0, 37°C for 48 hours. The mixture was then exhaustively dialyzed against acetic acid, urea containing mercaptoethanol and water, and freeze-dried, as described in

section C.

E. Optimization techniques

To avoid the need to execute a large number of experiments, which increases exponentially as the number of potentially influential factors increases when one uses full factorial experimental designs, 2-, 3- and 4-level fractional factorial experiments (Taguchi, 1957; Box and Hunter, 1961; U.S. National Bureau of Standards, 1957) were used to select influential factors and interactions in the reaction conditions for carbodiimide coupling (see Appendix I). Effect curves were plotted for significant factors by calculating averages of the data at each level of the factor and confidence limits were calculated as $(\pm \frac{t}{\sqrt{\text{no. of data}}} \sqrt{\frac{S_e}{df_e}})$

where t = values for t -distribution for a two-tailed t -test at the specified level of confidence (generally 90, 95 or 99% confidence) at degrees of freedom for error; S_e = sum of squares value for error; df_e = degrees of freedom for error.

Estimated relative lysine or threonine increases under favorable reaction conditions were calculated as

$$\hat{\mu} = X_1 + X_2 + X_3 + \dots + X_n - (n-1)(\mu)$$

where $X_1 \dots X_n$ are the averages at the most favorable level of each significant reaction condition, and μ is the overall average. The confidence limit for this estimate was calculated as $(\pm \frac{t}{\sqrt{NR}} \sqrt{\frac{S_e}{df_e}})$, where NR =

effective repeated number = total numbers of experiments divided by the sum of df used to estimate $\hat{\mu}$.

The simplex optimization method of Morgan and Deming (1974) was used in this study to find the optimal conditions, within the specified ranges allowed, for linking N^ε-benzylidene lysine to pepsin-hydrolyzed gluten (Appendix II).

F. Amino acid analysis

After first blocking cysteine residues by reaction with 4-vinyl pyridine, as described by Cavins et al. (1972), samples were hydrolyzed for amino acid analysis with p-toluenesulfonic acid in the presence of 3-(2-aminoethyl) indole for 24 hours at 110°C according to Liu and Chang (1971). Amino acids were analyzed on a single column system (Durrum Chem. Corp., Palo Alto, Calif.) attached to a Phoenix model M 6800 amino acid analyzer (Phoenix Precision Instrument Co., Philadelphia, Penna.).

Unless otherwise specified, lysine content and threonine content of samples determined by amino acid analysis were expressed either as g/100g total amino acids or as "relative lysine increase" and "relative threonine increase", respectively, defined here as the ratio of lysine or threonine content in the sample to the respective amino acid content in untreated vital gluten.

G. Nitrogen determination

Nitrogen content was determined on a Technicon Auto Analyzer II system, after prior digestion of samples by the micro-Kjeldahl digestion method of Concon and Soltess (1973). For conversion from nitrogen to protein content, factors of 5.70, 6.25 and 6.38 were used for gluten,

egg and casein samples, respectively.

H. In vitro "DNBS-available" lysine determination

Free ϵ -amino groups of samples were measured by the dinitro-benzenesulfonate (DNBS) spectrophotometric method of Concon (1975) as modified by Holguin and Nakai (1980). These values were reported as "DNBS-available" lysine content (g/100g).

I. In vitro "pepsin-pancreatin-digestible" lysine determination

Digestibility of samples was determined by amino acid analysis after an in vitro digestion with the enzymes pepsin and pancreatin according to the method of Stahmann and Woldegiorgis (1975), with the following modifications (Holguin and Nakai, 1980): after digestion, to 5.0 ml of the digestion mixture was added 5.0 ml of 14% sulfosalicylic acid; the mixture was then shaken for 15 – 30 min, pH adjusted to 2.2 with 6N NaOH, and volume adjusted to 15 ml with pH 2.2 citrate buffer. The sample was then centrifuged (1000 x g, 30 min), filtered through Whatman No. 1 filter paper and then through a sintered glass ultrafine filter and stored frozen until analyzed. After correction for the enzyme blank values, lysine content thus determined was reported as "pepsin-pancreatin-digestible" lysine (g/100g released amino acids). Composition of the other amino acids released by digestion was also analyzed simultaneously as g/100g released amino acids. As the digestion is incomplete, by taking into account the nitrogen content of samples, the amount of individual amino acid released per 100g sample may also be calculated and reported as g amino acid released/100g sample.

J. *Tetrahymena pyriformis* W bioassay for relative nutritive value determination

Tetrahymena pyriformis W culture was routinely maintained at 27°C in the dark in *Tetrahymena* medium (5g proteose peptone, 5g tryptone, 0.2g K_2HPO_4 , 1 litre distilled water) and transferred every 3 – 4 days. The culture was also maintained on Haskins agar (8.0g dextrin, 0.6g sodium acetate, 5.0g yeast extract, 0.6g liver extract, 5.0g tryptone, 16g agar, 1 litre distilled water) at pH 7.2 – 7.4 with sterile water as overlay; this culture was kept at 22°C in the dark and transferred every 30 days. For the assay, a 3-day old broth culture was centrifuged twice (3000 x g, 10 min), the pellet being resuspended in fresh 0.067M phosphate buffer (pH 7.2) each time.

Samples were predigested either with papain according to Evans et al. (1978, 1979) or with pepsin according to Evancho et al. (1977). Predigested samples were neutralized to pH 7.1 and frozen for analysis.

Vitamin stock solution A, mineral stock solutions B and C, buffer solution D, nucleotide solution E and dextrin solution G were prepared according to the original method of Stott et al. (1963) as modified by Evancho et al. (1977). Aliquots of predigested samples were used to provide 0.3mg N/ml of final culture medium. Assay procedures were as described by Evancho et al. (1977).

Tetrahymena growth was determined on 4-day old cultures by either direct microscopic count or by the 2,3,5-triphenyltetrazolium chloride reduction test. For direct microscopic count, an aliquot of the culture suspension was transferred to preserving fluid as described by Evancho et al. (1977) and organisms were counted on a J.F.Hartz double cell haemocytometer. The mean number of organisms per 1 mm square

for eight alternate 1 mm squares gave the test culture population in units of 10^4 organisms per ml. Relative nutritive values were calculated as (organism count for sample/organism count for ANRCcasein) X 100.

The enzymatic reduction of colorless 2,3,5-triphenyltetrazolium chloride (TPTZ) to dark red colored triphenylformazan (TPF) was also used to determine growth of the test culture populations. The method was essentially that originally described by Pilcher and Williams (1954), as modified by Wang et al. (1979). Aliquots of test culture populations which were autoclaved at 121°C for 20 minutes acted as the corresponding blanks for the test.

K. Baking procedure for enriched glutens

Model baking studies were conducted to investigate the effects of heating lysine enriched glutens in the absence or presence of a reducing sugar. Covalently lysine enriched gluten samples were formed into "doughs" by mixing 3g of the freeze-dried samples with water (76% w/w), with or without addition of 10% (w/w) glucose. Corresponding samples enriched by simple addition were prepared similarly, after thoroughly mixing and grinding with mortar and pestle dry mixtures of gluten and lysine or lysine derivative in the appropriate ratio. These doughs were baked at 190°C for 10 minutes. The resulting "breads" were cooled, separated into crust and crumb fractions and freeze-dried.

Baked samples were compared with respect to total lysine content (section F), DNBS-available lysine content (section H), pepsin-pancreatin-digestible lysine content (section I), Tetrahymena pyriformis W relative

nutritive value (section J) and color (section L).

L. Measurement of color in baked samples by spectrophotometric evaluation of extracts

Clear extracts of the brown pigments formed during baking were obtained by enzymatic digestion followed by precipitation and filtration. The methods of Tinkler et al. (1955) and Choi et al. (1949) were modified to give the following procedure:

To 0.1g ground sample in 5 ml pH 8 phosphate buffer was added 0.5 ml 3% Pronase solution. The mixture was incubated at 50°C for 10 minutes. After addition of 2 ml 60% trichloroacetic acid, the mixture was filtered on Whatman No. 1 filter paper with the aid of 0.2g celite filter aid. The filtrate was re-filtered if necessary to obtain a clear filtrate, and the absorbance was then read at 420nm against a reagent blank. (When Whatman No. 50 (hardened) filter paper was used, no filter aid or re-filtering was required to obtain a clear filtrate).

Unless otherwise specified, all procedures were carried out in duplicate.

RESULTS AND DISCUSSION

PART I. PREPARATION OF COVALENTLY ENRICHED GLUTEN BY THE CARBODIIMIDE REACTION

The carbodiimide reaction couples carboxyl and amino groups to form an amide bond. Since amino acids contain at least one amino (α -NH₂) and one carboxyl (α -COOH) group, they may participate in the reaction either as the nucleophile (amino group) or as the acylating agent (carboxyl group) to form an α - α amide bond, commonly known as a peptide bond. The bifunctional nature of amino acids therefore leads to various different peptides consisting of different combinations or order of linkage of the amino acids. In addition, some amino acids possess an additional carboxyl group in their side chains (eg. β - or γ -COOH of aspartic and glutamic acids, respectively), while others possess a side chain amino group (eg. ϵ -NH₂ of lysine), which may also participate in amide bond formation. The term isopeptide bond is commonly used to denote these amide bonds which are not formed by α - α coupling.

In peptide synthesis for fundamental research, where it is crucial to obtain a single product and to avoid formation of mixtures which would require further tedious isolation and purification techniques, protecting groups are used on some functional groups to ensure specificity of the reaction. Each reactant possesses only one functional group available for reaction. After completion of coupling to form the desired peptide bond, protecting groups are selectively removed.

In this work, little attention has been paid to the isolation of a

single well-defined product. Instead, emphasis has been placed on the digestibility and availability of the limiting amino acids which are covalently attached through amide bond formation to gluten. Although it may be safe to assume that the amino acid attached by a peptide bond should be readily digestible and available, the same may not hold true for the amino acid attached via an isopeptide bond. Thus, various gluten preparations and amino acid derivatives were investigated as potential starting materials for the reaction.

Vital wheat gluten has relatively low contents of amino (α - or ϵ -) and carboxyl (α -, β - or γ -) groups available as reactants for amide bond formation. At the same time, gluten is highly insoluble in water, forming a cohesive and elastic mass of dough, thus the few functional groups which are present may not be well exposed for reaction with the amino acid to be incorporated. Solubilization by sodium stearate addition (Kobrehel and Bushuk, 1977) may enhance exposure of these sites. Solubilization by pepsin hydrolysis (Oka *et al.*, 1965; Yang and McCalla, 1968) would not only improve aqueous dispersibility of gluten, but also increase the α -NH₂ and α -COOH contents. Solubilization by mild acid treatment designed to selectively hydrolyze amide groups of gluten (Wu *et al.*, 1976) would increase γ -COOH content as well as aqueous dispersibility.

Threonine contains only α -NH₂ and α -COOH groups for the reaction since its side chain hydroxyl group is not an active participant in carbodiimide reactions. However, lysine does contain an ϵ -amino group which may take part in the reaction. To promote α - α bond formation, ϵ -amino protecting groups may be used and selectively removed if desired

after the peptide bond formation is complete.

In this work, products enriched via primarily peptide bonds as well as products enriched via peptide and isopeptide bonds were prepared by using various starting materials. These products were then compared for extent of amino acid incorporation as well as product yield or recovery.

1. Attachment of N^ε-benzylidene lysine to pepsin-hydrolyzed gluten

Trifluoroacetyl, formyl, benzylidene and tert-butyloxycarboxyl groups were investigated by Holbek (1976) as possible protecting groups for the ε-amino group of lysine. Of these, only the N^ε-benzylidene lysine was incorporated during the carbodiimide reaction. No significant lysine increase was observed when any of the other three derivatives were used. Therefore, in the present study, further experiments were carried out to optimize the conditions for carbodiimide-mediated coupling of N^ε-benzylidene lysine to pepsin-hydrolyzed gluten via primarily α - α peptide bond formation. The benzylidene group was cleaved off by mild acidic conditions and ether extraction after the carbodiimide reaction.

A two-level fractional factorial experimental design (Appendix I-1a) was used to select influential factors in the reaction between pepsin-hydrolyzed gluten and N^ε-benzylidene lysine. The seven factors under consideration are shown in Table 4. As indicated in Table 5, within the chosen range of levels of these factors, only pH and pepsin hydrolysis time were significantly influential ($P < 0.05$) on the extent of lysine incorporation, the conditions of lower pH (6.4) and longer pepsin hydrolysis time (16 hours) being more effective.

Table 4. Factor levels for two-level fractional factorial experiment for the reaction between pepsin-hydrolyzed gluten and N^ε-benzylidene lysine.

factor	level	
	1	2
gluten concentration, % ^a	1	5
reaction time, hours	6	21
reaction temp., °C	40	65
pH	6.4	8.1
pepsin hydrolysis, hours	2	16
EDC, mg	200	270
N ^ε -benzylidene lysine, mg	200	468

^aLevel 1 = 500 mg/50 ml; Level 2 = 500 mg/ 10 ml.

Table 5. Analysis of variance of results of two-level fractional factorial experiment for the reaction between pepsin-hydrolyzed gluten and N^ε-benzylidene lysine (Dependent variable = lysine increase).

factor	SS	df	MS	F ^a
pepsin hydrolysis time	4.931	1	4.931	11.36*
pH	4.794	1	4.794	11.05*
temperature	0.019	1	0.019	0.044
N ^ε -benzylidene lysine	0.088	1	0.088	0.21
EDC amount	0.003	1	0.003	0.007
time	0.645	1	0.645	1.49
gluten concentration	0.675	1	0.675	1.56
time x gluten concn	0.025	1	0.025	0.058
error	3.043	7	0.434	
total	14.223	15		

^aF_{(0.01)1, 7} = 12.23**; F_{(0.05)1, 7} = 5.59*.

The pH of the reaction medium is critical to provide the protonated form of the carbodiimide while leaving the carboxyl and amino functional groups unprotonated (Addy *et al.*, 1973a); extremes of pH may lower the effective concentration of carbodiimides as they react additively with water to form ureas (Kurzer and Douraghi-Zadeh, 1967). In these experiments, average relative lysine increases of 3.2-fold and 2.1-fold were obtained at pH 6.4 and pH 8.1, respectively.

Peptide bond cleavage by pepsin hydrolysis results in increased contents of α -amino and α -carboxyl groups, and is accompanied by an increase in gluten dispersibility (Yang and McCalla, 1968; Oka *et al.*, 1965). Thus, conditions of longer pepsin hydrolysis time increase the concentrations of amino and carboxyl groups available as attachment sites for the N^ε-benzylidene lysine; increased dispersibility of gluten may also improve attachment of N^ε-benzylidene lysine due to enhanced exposure of gluten sites for reaction. In these experiments, average relative lysine increases of 2.1-fold and 3.2-fold were obtained using gluten hydrolyzed with pepsin for two hours and for 16 hours, respectively.

Although a longer pepsin hydrolysis time favored lysine increase, it resulted in significantly ($P < 0.01$) lower product yield (Table 6). Average yields of 42% and 30% were obtained for the 2 hour and 16 hour hydrolyzed samples, respectively. Table 7 shows the results of amino acid analysis of a 38 hour pepsin-hydrolyzed gluten sample before and after dialysis, and the corresponding lysine-enriched sample obtained by the carbodiimide method, which involves dialysis to remove excess reagents and side reaction products. The dialyzed pepsin-hydrolyzed gluten and lysine enriched sample both show lower contents of several amino acids,

Table 6. Analysis of variance of results of two-level fractional factorial experiment for the reaction between pepsin-hydrolyzed gluten and N^ε-benzylidene lysine (Dependent variable = product yield).

factor ^a	SS	df	MS	F ^b
pepsin hydrolysis time	0.0124	1	0.0124	75.78**
pH	0.0002	1	0.0002	1.22
N ^ε -benzylidene lysine	0.0004	1	0.0004	2.44
gluten concentration	0.0165	1	0.0165	100.83**
error	0.0018	11	0.00016	
total	0.0313	15		

^aThe sum of squares (SS) values for temperature, EDC amount and time were small and were incorporated into the error term.

^b $F_{(0.01)1, 11} = 9.65^{**}$; $F_{(0.05)1, 11} = 4.84^*$.

Table 7. Amino acid composition (g/100 g) of gluten, pepsin-hydrolyzed gluten and lysine enriched gluten samples.

	gluten	38 hour pepsin gluten	dialyzed 38 hr. pepsin gluten	N ^ε -benz.lys. 38 hr. pepsin gluten ^a	N ^ε -benz.lys. 1 hr. pepsin gluten ^b
asp	2.81	3.04	2.64	3.19	2.85
thr	2.23	2.50	2.62	3.11	2.30
ser	4.48	4.61	3.83	3.89	4.14
glu	37.45	37.07	39.51	33.58	36.89
pro	12.67	12.23	14.82	15.44	12.61
gly	2.88	3.06	2.98	2.29	2.55
ala	2.17	2.36	1.67	1.36	1.83
val	2.96	3.04	2.45	2.30	2.65
met	1.16	1.04	0.88	1.21	1.17
ile	3.15	3.32	2.46	2.29	2.37
leu	6.50	6.57	5.35	5.14	5.15
tyr	3.09	3.14	3.00	3.15	3.42
phe	4.86	4.86	4.21	4.35	4.81
lys	1.33	1.45	1.29	5.73	5.93
his	1.78	1.63	2.10	2.36	1.43
arg	3.00	2.90	2.88	3.03	2.91
trp	0.79	0.69	0.65	0.89	0.77
cys	1.68	1.50	1.65	1.70	1.60

^aExperimental conditions for preparation: pepsin hydrolysis time for solubilization, 38 hours; gluten concentration, 0.8% (500 mg/62.5 ml); EDC amount, 174 mg; N^ε-benzylidene lysine, 250 mg; NHS, 46 mg; pH 7.2; reaction temperature, 54°C; reaction time, 19 hours.

^bExperimental conditions for preparation: pepsin hydrolysis time for solubilization, 1 hour; gluten concentration, 2.5% (500 mg/ 20 ml); EDC, 289 mg; N^ε-benzylidene lysine, 188 mg; NHS, 50 mg; pH 5.7; reaction temperature, 40°C; reaction time, 6 hours.

such as alanine, valine, isoleucine and phenylalanine. These results imply that the low yield of product obtained when a long pepsin hydrolysis time is used for solubilization of gluten may be due to the production of small peptides or free amino acids which are lost during dialysis.

The major sites of hydrolytic action by pepsin from the gastric mucosa are at the carboxyl end of the amino acid residues tryptophan, phenylalanine, tyrosine, methionine and leucine, although various other minor sites of action exist for this enzyme of rather broad specificity (White et al., 1973). Yang and McCalla's work (1968) on the action of proteolytic enzymes on wheat gluten indicated increasing amounts of non-protein nitrogen and terminal amino nitrogen with increasing hydrolysis time from 2 to 48 hours, when pepsin at 1 or 2% level was used as the proteolytic enzyme. However, free amino acid nitrogen was not detected until 12 hours of hydrolysis, at which time free phenylalanine, leucine, valine and tyrosine were present. Autohydrolysis by naturally occurring enzymes in gluten was suggested to occur concurrently with hydrolysis by pepsin, and released appreciable amounts of leucine and phenylalanine as well as some hydrophilic amino acids. Proteolysis of glutenin by pepsin was shown by Oka et al. (1965) to rapidly decrease the viscosity of the dispersion and increase the water-soluble fraction. These workers suggested that these early changes were results of cleavage of a few peptide bonds in glutenin; further pepsin action would cause a substantial increase in small peptide fragments.

Based on these observations, a shorter hydrolysis time of 1 hour was then attempted for solubilization of gluten prior to lysine attachment. Dispersions (0.5, 2.0 and 5.0%) of gluten all showed visible increase in

dispersibility and decrease in viscosity within 15 minutes of addition of pepsin at a pepsin:gluten ratio of 1:100 (w/w) at pH 2, 37°C. After 1 hour, samples were neutralized to pH 7. The 2 and 5% dispersions showed some tendency for sedimentation after neutralization while the 0.5% sample remained well dispersed. Therefore, for further studies, 0.5% dispersions of gluten were solubilized using 0.005% pepsin at pH 2, 37°C for 1 hour, followed by neutralization to pH 7 and freeze-drying.

Table 7 indicates that the lysine-enriched sample obtained using the 1 hour hydrolyzed gluten had an amino acid composition more similar to that of untreated gluten than the enriched 38 hour hydrolyzed gluten, except for lower isoleucine and leucine contents. The yield of product remained relatively low, usually between 45% and 60%, but was still a significant improvement over the samples prepared after long pepsin hydrolysis times.

Optimization of reaction conditions was attempted by the simplex method (Appendix II) using a relatively short (1 hour) pepsin hydrolysis time for gluten solubilization. The variable factors and their set boundaries were pH (4.5 - 10.0), N^ε-benzylidene lysine amount (100 to 500mg) and EDC amount (100 to 500mg). The reaction conditions which were held constant were pepsin-hydrolyzed gluten concentration (2.5%, using 500mg/20 ml), NHS amount (50mg), reaction temperature (40°C) and reaction time (6 hours). The factor levels at each vertex and results of the simplex search are shown in Table 8.

Figure 3 shows the relative increase in lysine content as a function of the simplex search. The leveling off in response at about four-fold increase in lysine content indicates that the search has homed in on the

Table 8. Results of simplex search for reaction between N^ε-benzylidene lysine and 1 hour pepsin hydrolysate of gluten.

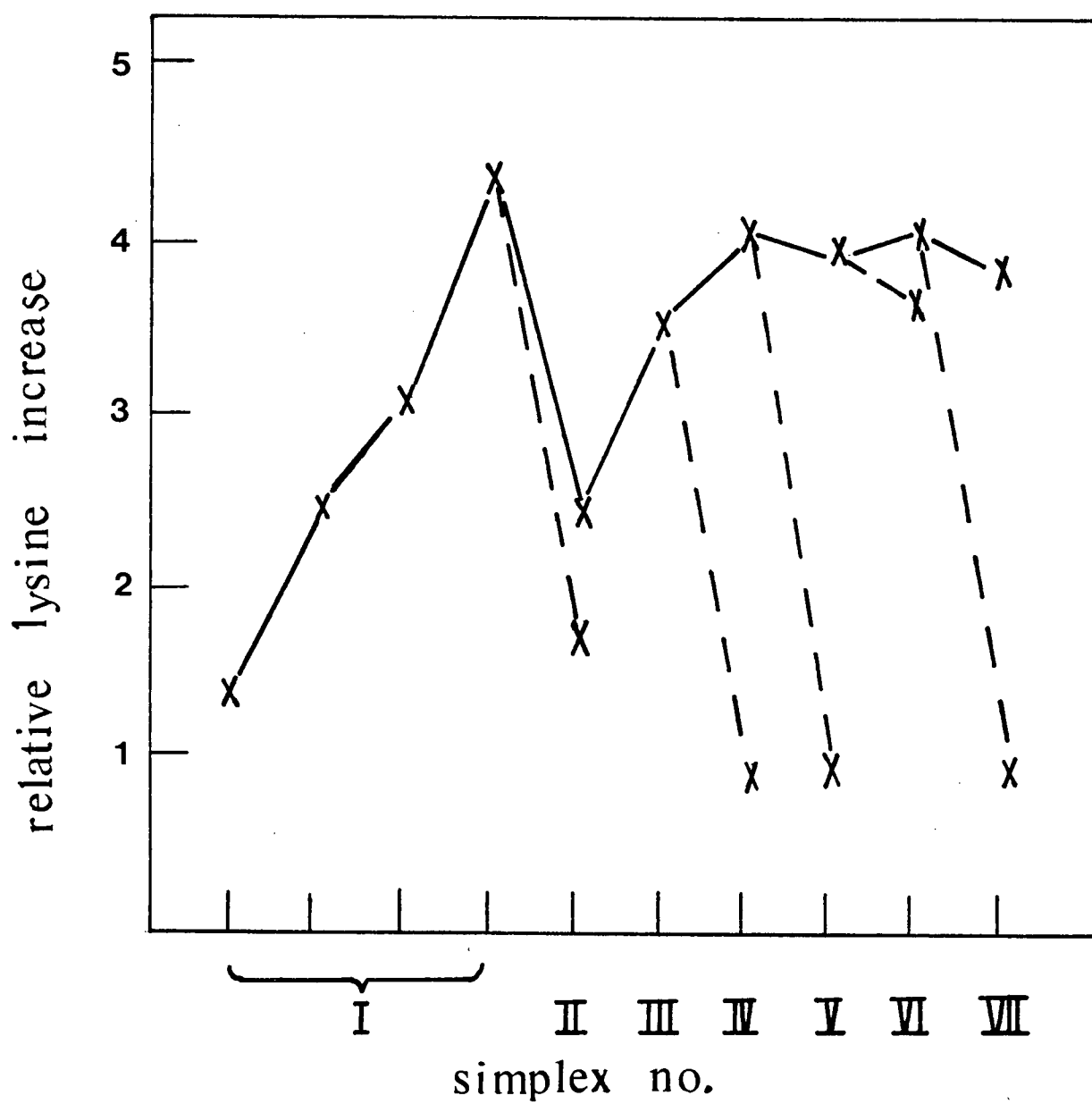
vertex number	simplex number	vertices retained	Factor levels ^a			relative lysine increase
			pH	mg N ^ε -benz.lys.	mg EDC	
1			4.50	100	100	1.30
2			9.70	188	144	2.40
3			5.70	477	144	3.10
4			5.70	188	289	4.40
5	II	2, 3, 4	9.56	468	285	1.70
6		2, 3, 4	8.30	376	238	2.30
7	III	2, 3, 4	5.77	193	147	3.50
8	IV	3, 4, 7	1.74	384	240	1.00 ^b
9		3, 4, 7	7.71	237	168	4.10
10	V	4, 7, 9	7.08	-65	260	1.00 ^b
11		4, 7, 9	6.05	341	172	3.90
4'			5.70	188	289	4.33 ^c
12	VI	4, 9, 11	7.21	317	273	3.70
13		4, 9, 11	6.13	224	178.5	4.10
14	VII	4, 9, 13	6.97	91	252	1.00 ^b
15		4, 9, 13	6.74	153.5	232	3.83

^aThe boundary conditions were as follows: pH: 4.5 to 10.0; N^ε-benzylidene lysine: 100 to 500 mg; EDC: 100 to 300 mg.

^bViolated boundary condition; a response of 1.00 was arbitrarily assigned to force the factor back inside the boundary by a contraction.

^cThe response for vertex 4 was checked after it had occurred in (k + 1) or 4 successive simplexes.

Figure 3. Relative lysine increase by the carbodiimide reaction as a function of simplex number.



optimum conditions for the reaction, within the limits of the set boundaries. The values for the centroid of the last complete simplex are pH 6.39, 248mg of N^ε-benzylidene lysine and 202 mg of EDC. The lysine increase obtained under these experimental conditions was 3.80.

Multiple regression analysis of the combined data of the two-level fractional factorial experiment (described in Table 4) containing sixteen data and an initial simplex containing eight vertices was used to obtain an approximation of the optimum reaction conditions for maximal lysine increase (Table 9). The boundary conditions for the simplex were pepsin hydrolysis time (3 - 48 hours), pH (4.5 - 10.0), reaction temperature (25 - 60°C), N^ε-benzylidene lysine amount (50 - 500 mg), EDC amount (50 - 200 mg), reaction time (1 - 24 hours) and pepsin-hydrolyzed gluten concentration (1 - 5%). Using these seven factors as the independent variables X_1 to X_7 , respectively, and with ten additional independent variables consisting of the interactions X_4X_5 , X_5X_7 , X_4X_7 and $(X_i)^2$, stepwise multiple regression yielded the following equation:

$$\begin{aligned} \text{Relative lysine increase } Y = & -10.92 + 0.12X_1 + 3.63X_2 + \\ & 0.055X_3 - 0.002X_4 + 0.002X_5 - 0.005X_6 - 0.32X_7 + 0.00002X_4X_5 - \\ & 0.0005X_4X_7 + 0.003X_5X_7 - 0.002X_1^2 - 0.28X_2^2 - 0.0005X_3^2 - \\ & 0.00003X_5^2 + 0.001X_6^2 \\ & (R = 0.906; F\text{-probability level} = 0.05; X_4^2 \text{ and } X_7^2 \text{ were linear} \\ & \text{combinations of variables included in the regression}) \end{aligned}$$

Solving the simultaneous equations obtained by partial derivatization of the above regression equation yielded the following values for the reaction conditions: pepsin hydrolysis time, 30 hours; pH 6.48; reaction temperature, 55°C; N^ε-benzylidene lysine, 140 mg; EDC, 130 mg; reaction

Table 9. Reaction conditions and corresponding lysine increase used for multiple regression analysis to maximize relative lysine increase.

expt. no. ^a	reaction conditions							Relative lysine increase
	pepsin hydrolysis time, hr.	pH	temp. °C	N ^ε -benz. lysine, mg.	EDC, mg.	reaction time, hr.	protein conc. %	
1	2.0	6.4	40	200	200	6	1.0	2.43
2	16.0	8.1	40	468	270	6	1.0	1.97
3	2.0	6.4	65	468	270	6	1.0	2.42
4	16.0	8.1	65	200	200	6	1.0	1.91
5	16.0	6.4	40	468	200	21	1.0	3.95
6	2.0	8.1	40	200	270	21	1.0	1.76
7	16.0	6.4	65	200	270	21	1.0	2.76
8	2.0	8.1	65	468	200	21	1.0	2.18
9	2.0	6.4	40	200	200	6	5.0	2.12
10	16.0	8.1	40	468	270	6	5.0	2.95
11	2.0	6.4	65	468	270	6	5.0	2.71
12	16.0	8.1	65	200	200	6	5.0	2.90
13	16.0	6.4	40	468	200	21	5.0	4.29
14	2.0	8.1	40	200	270	21	5.0	1.82
15	16.0	6.4	65	200	270	21	5.0	4.73
16	2.0	8.1	65	468	200	21	5.0	1.14
17	3.0	4.5	25	50	50	1	1.0	0.84
18	42.5	5.4	31	127	76	5	1.7	2.37
19	10.5	9.3	31	127	76	5	1.7	1.23
20	10.5	5.4	56	127	76	5	1.7	2.40
21	10.5	5.4	31	445	76	5	1.7	1.71
22	10.5	5.4	31	127	182	5	1.7	2.24
23	10.5	5.4	31	127	76	21	1.7	2.41
24	10.5	5.4	31	127	76	5	4.5	1.83

^aExperiments 1 to 16 were from a fractional factorial design; experiments 17 to 24 were the eight vertices from an initial simplex for the 7 factors in the reaction conditions.

time, 2.5 hours; protein concentration, 1%. The estimated optimal and experimental values for the lysine increase resulting from the carbodiimide reaction under the above reaction conditions were 3.99 and 3.89, respectively. This result confirms that within the range of current experimental conditions, approximately four-fold increase in lysine content can be achieved.

2. Attachment of N^ε-acetyl lysine to gluten

Preliminary selection of influential factors in the carbodiimide mediated reaction between N^ε-acetyl lysine and gluten was based on a two-level fractional factorial experiment (Appendix I-1b). The factors of interest and their assigned levels are shown in Table 10, and the analysis of variance of the results is shown in Table 11. Of the eight factors under consideration, only reaction time was found to be significant at $P < 0.05$ in influencing the relative lysine increase. N^ε-Acetyl lysine amount and pH were significant at $P < 0.10$. Within the chosen levels of these factors, 2 hour reaction time, 200 mg N^ε-acetyl lysine and pH 5 were the more favorable conditions for N^ε-acetyl lysine attachment. For the other factors, the condition of 5% gluten concentration gave a significantly higher relative lysine increase ($P < 0.25$), while the levels of sodium stearate amount, activation time, EDC amount and reaction temperature were non-significant. The estimated relative lysine increase from reaction under these more favorable conditions is 2.7 ± 0.6 .

In these experiments, the relative lysine increase obtained ranged from no increase to a 3-fold increase, with an average increase of 1.8-fold. The 3-fold increase was obtained under the following reaction conditions:

Table 10. Factor levels for 2-level fractional factorial experiment for reaction between N^ε-acetyl lysine and gluten.

factor	level	
	1	2
gluten conc., %	1 (250 mg/25 ml)	5 (250 mg/5 ml)
sodium stearate, mg.	0	25
EDC amount, mg.	50	250
pH	5	8
reaction time, hr.	2	24
activation time, min.	0	30
reaction temp, °C	22	40
N ^ε -acetyl lysine, mg.	50	200

Table 11. Analysis of variance of results from 2-level experiment for reaction between N^ε-acetyl lysine and gluten.

factor ^a	SS	df	MS	F
gluten conc.	0.345	1	0.345	2.61
sodium stearate	0.179	1	0.179	1.35
pH	0.566	1	0.566	4.28
reaction time	0.936	1	0.936	7.07*
activation time	0.196	1	0.196	1.48
N ^ε -acetyl lysine	0.597	1	0.597	4.51
gluten x stearate	0.328	1	0.328	2.48
error	1.058	8	0.132	
total	4.205	15		

$F_{(0.01)1, 8} = 11.3^{**}$; $F_{(0.05)1, 8} = 5.32^{*}$; $F_{(0.10)1, 8} = 3.46$;

$F_{(0.25)1, 8} = 1.54$.

^aThe sum of squares (SS) values for EDC amount and reaction temperature were incorporated into the error SS since F values were nonsignificant ($P > 0.25$).

5% gluten (250 mg/5 ml), 250 mg EDC with an activation time of 30 min, 200 mg N^ε-acetyl lysine, pH 5 and 2 hour reaction time at 22°C.

It is interesting to note that the addition of sodium stearate did not favor the reaction. Sodium stearate enhances solubilization of gluten in water (Kobrehel and Bushuk, 1977) and it was hoped that its presence would encourage N^ε-acetyl lysine attachment to gluten by increasing the surface area of soluble gluten available for reaction. However, although samples containing stearate were initially much more dispersible in water than samples without stearate (the latter formed dough-like masses), almost all samples formed clumps of insoluble matter after the subsequent addition of EDC, regardless of the presence or absence of stearate. It is possible that the carboxylate group of stearate may have reacted with the carbodiimide, effectively reducing the concentrations of both stearate for solubilization and EDC for the coupling reaction. This would explain the loss of dispersibility even in stearate-containing samples after EDC addition, as well as the higher extent of N^ε-acetyl lysine attachment to samples which initially contained no stearate.

In order to analyze in further detail the reaction conditions for N^ε-acetyl lysine attachment, a four-level fractional factorial experiment was used to study the three most influential factors – pH, reaction time, and N^ε-acetyl lysine amount (Appendix I-3a). The assigned levels of the factors are shown in Table 12. Relative lysine increases ranged from no increase to 1.9-fold increase, with an average of 1.5-fold increase. The highest increase was obtained under the following reaction conditions: 5% gluten (250 mg/5 ml), 50 mg EDC, 50 mg N^ε-acetyl lysine, pH 5 and reaction time of 1 hour at 22°C.

Table 12. Factor levels for 4-level_ε fractional factorial experiment for reaction between N^ε-acetyl lysine and gluten.

factor	level			
	1	2	3	4
pH	5	6	7	8
N ^ε -acetyl lysine, mg.	50	100	200	400
reaction time, hours	1	2	6	24

The analysis of variance of the results is shown in Table 13. Only pH was found to have a significant ($P < 0.25$) influence on the relative lysine increase. Figure 4 illustrates this pH effect. Among the chosen levels from pH 5 to 8, the attachment of N^ε-acetyl lysine to gluten was at a maximum at pH 5 and at a minimum at pH 7. These results again confirm the importance of pH in the carbodiimide reaction, due to its effect on protonation or dissociation of reactive groups and on the stability of EDC.

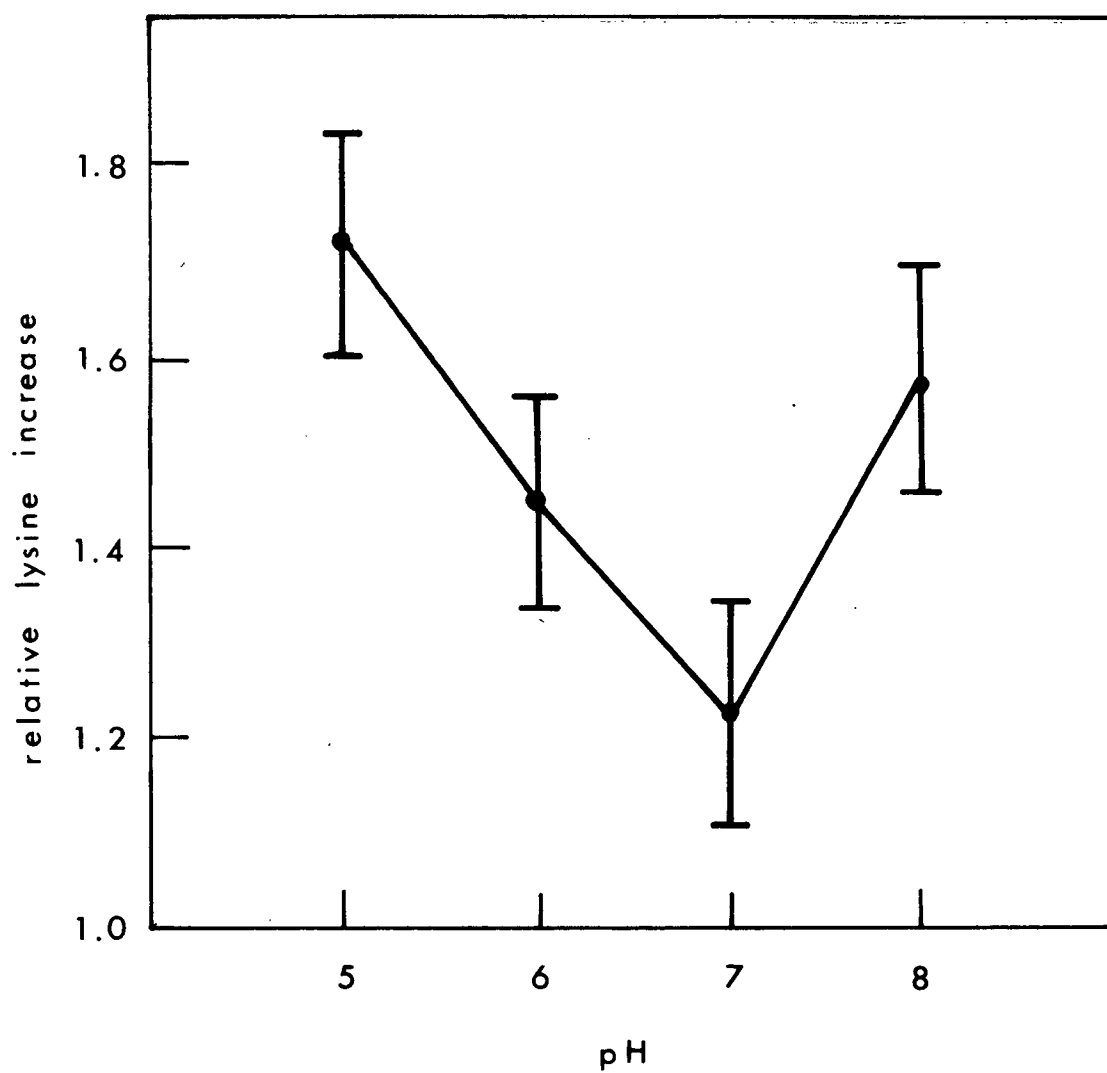
The four-level fractional factorial experiment was chosen to give further details on the conditions found by the two-level experiment to affect the reaction. In general, fractional factorial experiments are useful for screening many factors in order to detect a few significant ones, if it is presumed that some of the interactions between factors can be assumed a priori to be insignificant (Davies, 1967). The four-level ($L_{16}(4^5)$) experiment is designed to confirm optimization when fewer than four factors are significant in the two-level ($L_{16}(2^{15})$) experiment, with reasonably low degrees of interactions (Taguchi, 1957). However, the choice of levels is very important – as the range becomes narrower, the magnitudes of the effects may become smaller and thus the effects are more likely to be masked by experimental error; on the other hand, if the range chosen shows very large effects, analysis may again be complicated as the large effects can give rise to appreciable interactions (Davies, 1967). This may explain why some of the factors found to be significant by the two-level experiments were insignificant in the four-level experiments. It is also possible that some higher-order interactions are playing important roles but were not detected by the fractional factorial experiments due to confounding with main effects and with interactions of a lower order.

Table 13. Analysis of variance of results from 4-level fractional factorial experiment for reaction between N^ε-acetyl lysine and gluten.

factor	SS	DF	MS	F
pH	0.537	3	0.178	2.97*
N ^ε -acetyl lysine	0.162	3	0.053	0.88
reaction time	0.212	3	0.070	1.17
error	0.358	6	0.060	
total	1.269	15		

$$F_{(0.10)3, 6} = 3.29^{**}; \quad F_{(0.25)3, 6} = 1.78^*$$

Figure 4. Effect of pH on the attachment of N^ε-acetyl lysine to gluten.



3. Comparison of lysine versus N^ε-protected derivatives and gluten versus pepsin-hydrolyzed gluten for the carbodiimide reaction

The effects of firstly, different ϵ -amino substituents of lysine and secondly, solubilization of gluten by pepsin hydrolysis were compared to determine the better choice of reactants for the reaction in terms of effectiveness of lysine attachment and product yield. Table 14 shows the relative lysine increase and product yield obtained by reaction of lysine, N^ε-acetyl lysine or N^ε-benzylidene lysine with gluten or pepsin-hydrolyzed gluten.

4.0-fold and 6.5-fold increases in lysine content resulted after reaction of pepsin-hydrolyzed gluten with N^ε-benzylidene lysine and N^ε-acetyl lysine, respectively. However, these increases were accompanied by low product yields (47% and 58%). As suggested by the work on reaction of N^ε-benzylidene lysine and pepsin-hydrolyzed gluten (Results and Discussion Part I-1), although prolonged pepsin hydrolysis favors the coupling reaction, it results in significantly lower yields, probably as a result of the production of small peptides or free amino acids which are lost during dialysis. Despite the present use of a relatively short (1 hour) hydrolysis time for gluten solubilization, the yields of product remained low.

When untreated gluten was used as the reactant, product yields ranging from 90 - 95% were achieved. However, the extent of lysine incorporation was decreased in these products compared to that accomplished using pepsin-hydrolyzed gluten, probably as a result of the decrease in exposed surface area and lower contents of α -NH₂ and α -COOH groups available as reaction sites in the insoluble gluten samples.

Table 14. Relative lysine increase and product yield obtained by the reaction of lysine, N^ε-benzylidene lysine or N^ε-acetyl lysine with gluten or pepsin-hydrolyzed gluten.^a

reactants	relative lysine increase	product yield, %
gluten lysine	2.0	95
gluten N ^ε -benzylidene lysine	1.6	92
gluten N ^ε -acetyl lysine	2.5	94
1 hr. pepsin-hydrolyzed gluten N ^ε -benzylidene lysine	4.0	47
1 hr. pepsin-hydrolyzed gluten N ^ε -acetyl lysine	6.5	58

^aReaction conditions: 2.5% gluten or hydrolyzed gluten (500 mg/20 ml); 200 mg lysine/lysine derivative; 300 mg EDC; 50 mg NHS; pH 5.7; 40°C for 6 hours.

1.6-fold, 2.0-fold and 2.5-fold increases were attained by reaction of gluten with N^ε-benzylidene lysine, lysine and N^ε-acetyl lysine, respectively.

The reaction of gluten with N^ε-acetyl lysine gave higher relative lysine increases than the reaction with either lysine or N^ε-benzylidene lysine. The reaction of pepsin-hydrolyzed gluten with N^ε-acetyl lysine also gave higher relative lysine increase than the reaction with N^ε-benzylidene lysine. The size, charge and spatial orientation of the side chain of the amino acid are expected to influence the approach of the amino and carboxyl components for the coupling reaction to form a peptide bond. Addy *et al.* (1973b) reported a general increase in yield of incorporation of amino acid with increasing size of the aliphatic side chain of the amino acid. The large size and hydrophobicity of the benzylidene substituent may have prevented proper alignment of groups required for the reaction.

4. Attachment of lysine or N^ε-benzylidene lysine to acid-solubilized gluten

The aqueous dispersibility of gluten can be improved by mild acidic hydrolysis (Wu *et al.*, 1976). By careful control of the hydrolytic conditions, solubilization occurs primarily as a result of conversion of amide groups of glutamine residues to carboxyl groups, with a minimum alteration of peptide bonds. Wu *et al.* (1976) reported amide nitrogen contents of 280, 23.2, 205.7 and 249.1 mM per 100 g protein for untreated gluten and gluten treated at 121°C for 15 minutes with 0.5N HCl, 0.07N HCl and 0.02N HCl, respectively. The attachment of lysine to 0.5N HCl and 0.05N HCl solubilized gluten using carbodiimide was compared.

Table 15 indicates that 5.5-fold increase in lysine was achieved by reaction of 0.5N HCl gluten with lysine using rather mild conditions and relatively low molar excess of reagents. Up to 20-fold increase could be attained when larger amounts of lysine were present in the incubation mixture with 0.5N HCl gluten, whereas only 4.8-fold increase resulted under similar reaction conditions using 0.05N HCl gluten. These results imply that the reaction is favored using gluten solubilized by more severe acid hydrolysis. It is possible that the 0.5N HCl gluten contains higher contents of γ -COOH and possibly also more α -COOH and α -NH₂ groups which are available for coupling to lysine. The more severe acidic hydrolysis may also have led to denaturation resulting in easier accessibility to the functional groups for reaction. However, too drastic acid hydrolysis may be undesirable due to formation of intermolecular and intramolecular crosslinking between γ -COOH and α - or ϵ -NH₂ groups on gluten molecules, which may hinder digestibility either by insolubilization which can prevent proteolytic enzyme penetration or by masking of the sites of enzyme attack (Hurrell and Carpenter, 1977; Otterburn *et al.*, 1977).

N^ε-Benzylidene lysine could also be attached to 0.5N HCl gluten. Table 15 shows that a 3.9-fold increase in lysine content resulted by reaction under relatively mild conditions. This product would presumably contain lysine linked to gluten primarily by γ ($\begin{smallmatrix} \text{O} \\ \parallel \\ \text{C} \end{smallmatrix}$) - α (NH) isopeptide bonds, in contrast to the product obtained by reaction of lysine with 0.5N HCl gluten, which would contain lysine linked by γ - α as well as γ - ϵ isopeptide bonds.

These results demonstrate the feasibility of high levels of incorporation of lysine/lysine derivative through the carbodiimide reaction

Table 15. Preparation of enriched gluten by reaction of acid-solubilized gluten with lysine or N^ε-benzylidene lysine.

reactants	reaction conditions	relative lysine increase
0.5N acid gluten lysine	2.1% acid gluten (500 mg/24 ml); 50 mg EDC; 50 mg lysine; pH 5.0; 22°C for 2 hours.	5.5
0.5N acid gluten N ^ε -benzylidene lysine	2.3% acid gluten (500 mg/22 ml); 100 mg EDC; 15 min activation time; 100 mg N ^ε -benzylidene lysine; pH 8.5; 60°C for 5.5 hours.	3.9
0.5N acid gluten lysine	5% acid gluten (500 mg/10 ml); 190 mg EDC; 700 mg lysine; pH 6.0; 22°C for 4 hours.	20.0
0.05N acid gluten lysine	2.5% acid gluten (5000mg/200 ml); 2000 mg EDC; 5000 mg lysine; pH 6.0; 22°C for 4 hours	4.8

with gluten solubilized by rather mild conditions of acid hydrolysis. Product yields were high, being typically 80 - 90% of the starting material, gluten. Use of acid-solubilized gluten may have an added advantage in that the deamidation of glutamine residues in gluten or the gliadin fraction of gluten renders the protein no longer harmful to persons sensitive to celiac disease (Collins and Isselbacher, 1964).

5. Attachment of threonine to pepsin-hydrolyzed gluten

Possible influential factors in the carbodiimide-mediated attachment of threonine to gluten which had been solubilized by partial pepsin hydrolysis were studied using a two-level fractional factorial experiment (Appendix I-1c). The ten factors under consideration and their assigned levels are indicated in Table 16.

The results of the analysis of variance of these experiments are shown in Table 17, and indicate that the following factors were significant ($P < 0.01$) in influencing the relative threonine increase: EDC amount, gluten concentration, ionic strength, threonine amount and activation time. Within the chosen levels of these factors, threonine attachment to pepsin-hydrolyzed gluten was favored by high EDC and threonine addition (500 mg), high concentration of gluten (5%), no addition of NaCl and no activation time. High concentrations of the reactants (EDC, threonine and gluten) would be expected to encourage peptide bond formation by driving the reaction in the forward direction. High ionic strength may discourage reaction by decreasing solubility of gluten. Activation time or pre-incubation of gluten with EDC prior to threonine addition may reduce incorporation of threonine due to blocking of side chain carboxyl groups

Table 16. Factor levels for 2-level fractional factorial experiment for reaction between threonine and pepsin-hydrolyzed gluten.

factor	level	
	1	2
pepsin hydrolysis, hr.	1	24
EDC amount, mg.	100	500
gluten concn., %	1 (500 mg/50 ml)	5 (500 mg/10 ml)
pH	5	8
ionic strength, % NaCl	0	5
reaction time, hr.	2	24
threonine amount, mg.	100	500
reaction temperature, °C	22	40
activation time, min.	0	30
polarity, % ethanol	0	5

Table 17. Analysis of variance of results from 2-level experiment for reaction between threonine and pepsin-hydrolyzed gluten.

Factor ^a	SS	df	MS	F
pepsin hydrolysis time	0.051	1	0.051	2.53
EDC amount	2.641	1	2.641	130.96**
gluten concentration	1.626	1	1.626	80.63**
ionic strength	1.626	1	1.626	80.63**
threonine amount	0.601	1	0.601	29.80**
activation time	0.951	1	0.951	47.16**
gluten conc. x threonine	0.391	1	0.391	19.39**
reaction time x temp.	1.051	1	1.051	52.12**
pepsin hyd. x EDC	0.106	1	0.106	5.26
error	0.121	6	0.0202	
total	9.165	15		

$F_{(0.01)1, 6} = 13.75^{**}$; $F_{(0.05)1, 6} = 5.99^{*}$.

^aThe sum of squares (SS) values for pH, reaction time, reaction temperature and polarity were small and were incorporated into the error term.

which could otherwise participate in the reaction, or may result in hydrolysis of the O-acylisourea intermediate without peptide bond formation.

Table 17 also shows that the two-factor interactions between gluten concentration and threonine, and between reaction time and temperature were significant ($P < 0.01$), with the combinations of 500 mg threonine and 5% gluten, and of 22°C and 24 hours, favoring the reaction.

The estimated relative threonine increase from reaction under the conditions found to be favorable by these experiments is 3.1 ± 0.2 .

In these experiments, the relative threonine increase ranged from no increase to a 4-fold increase, with an average increase of 1.7-fold. However, in analogy to the attachment of lysine derivatives to pepsin-hydrolyzed gluten, product yields in these threonine experiments were also low, typically from 30 – 50%.

Subsequently, a four-level fractional factorial experiment was carried out in an attempt to optimize the levels of EDC and threonine amounts and pepsin-hydrolyzed gluten concentration for the reaction (Appendix I-3b). The assigned levels for the factors are shown in Table 18 and the analysis of variance is shown in Table 19. Only threonine amount was significant ($P < 0.05$) in influencing threonine attachment, with higher levels of threonine favoring the attachment (Figure 5). An average relative threonine increase of 2-fold was achieved but again product yields approached only 40 to 60%.

Table 18. Factor levels for 4-level fractional factorial experiment for reaction between threonine and pepsin-hydrolyzed gluten.

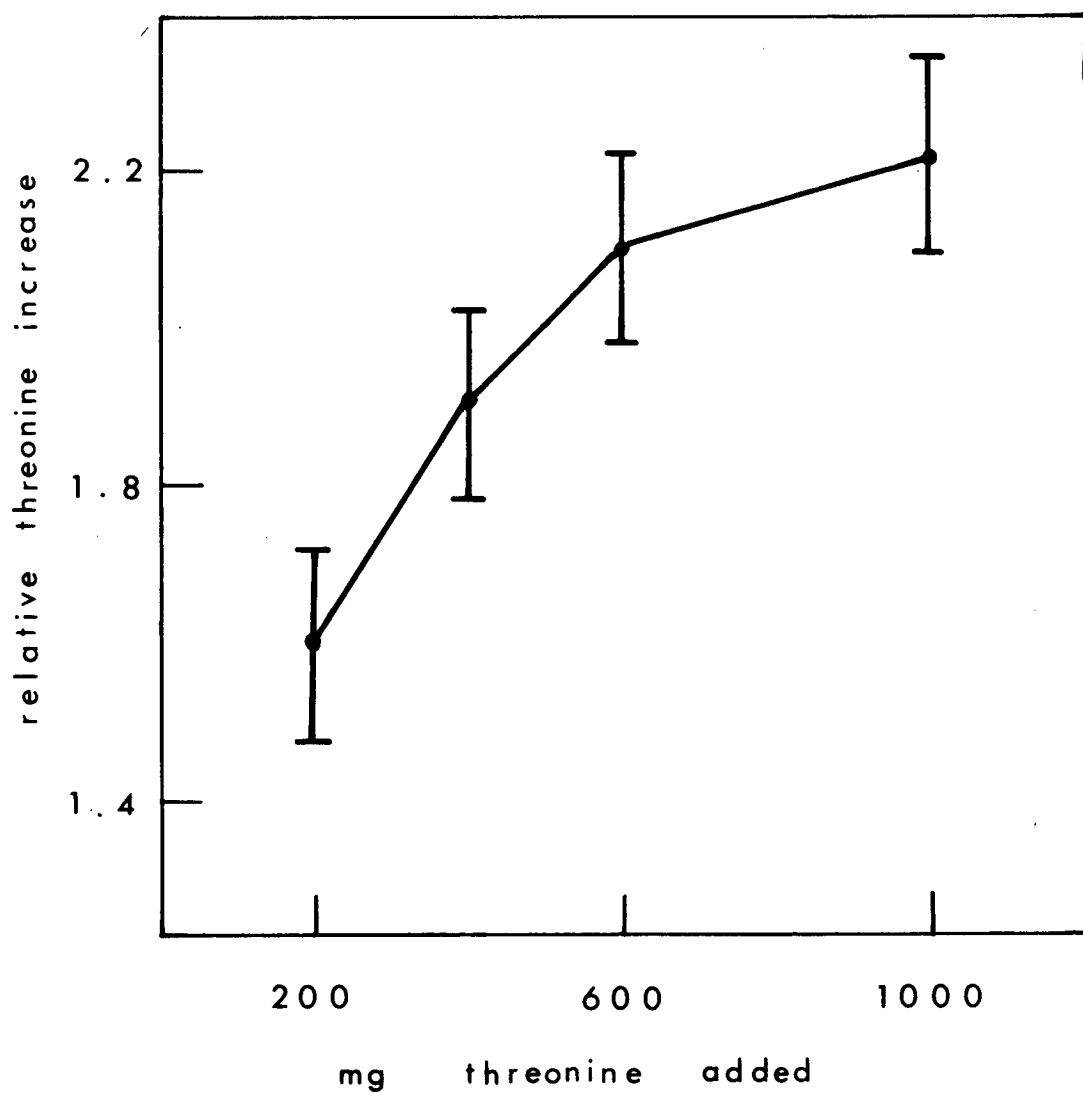
factor	level			
	1	2	3	4
EDC amount, mg	200	400	600	1000
threonine amount, mg	200	400	600	1000
gluten concentration, %	2.5	5.0	7.5	10.0

Table 19. Analysis of variance of results from 4-level fractional factorial experiment for reaction between threonine and pepsin-hydrolyzed gluten.

factor	SS	df	MS	F
EDC amount	0.0932	3	0.0310	0.684
threonine amount	0.9177	3	0.3059	6.748*
gluten concentration	0.0622	3	0.0207	0.457
error	0.2720	6	0.0453	
total	1.3451	15		

$$F_{(0.01)3, 6} = 9.78^{**}; \quad F_{(0.05)3, 6} = 4.76^*$$

Figure 5. Effect of level of threonine addition on the attachment of threonine to pepsin-hydrolyzed gluten



6. Attachment of threonine to gluten

Due to the poor product yields obtained in the reaction between threonine and pepsin-hydrolyzed gluten, the reaction was attempted using untreated vital gluten as the starting material. The effects of EDC and threonine amounts, gluten concentration, pH, reaction time and temperature, and polarity of the reacting medium were studied using a three-level fractional factorial experiment (Appendix I-2, and Table 20). The results of the analysis of variance of these data are shown in Table 21.

Of the factors considered, only gluten concentration and the two-factor interaction between EDC and threonine amounts were significant ($P < 0.01$) in affecting the attachment reaction. Figures 6 and 7 illustrate these effects. The influence of gluten concentration may be the effect of increasing the concentration of reactant (available amino and carboxyl groups) for the reaction. The relative threonine increase attainable was greater with increasing gluten concentration; a concentration of 5% was thus the most favorable among the levels considered in these experiments. The combination of 100 mg EDC with 400 mg threonine gave the highest relative threonine increase (Figure 7). This represents approximately a 6:1 molar ratio of threonine to EDC. The carbodiimide coupling reaction involves the initial build up of labile O-acylisourea intermediate, which in the absence of any nucleophilic species or base may simply be hydrolyzed in aqueous solution to regenerate the carboxyl group, or else may undergo a slow isomerization to the more stable N-acylurea (Hoare and Koshland, 1967; Perfetti et al., 1976). The addition of a nucleophile such as an amino group to the O-acylisourea intermediate

Table 20. Factor levels for 3-level fractional factorial experiment for reaction between threonine and gluten.

factor	level		
	1	2	3
EDC amount, mg.	100	200	400
threonine amount, mg.	100	200	400
gluten concentration, %	1 (250 mg/25 ml)	2.5 (250 mg/10 ml)	5 (250 mg/5 ml)
pH	5	6.5	8
reaction time, hr.	1	4	24
reaction temperature, °C	22	40	60
polarity	0	5% ether	5%ethanol

Table 21. Analysis of variance of results from 3-level experiment for reaction between threonine and gluten.

factor ^a	SS	df	MS	F
EDC amount	0.042	2	0.021	2.80
threonine amount	0.042	2	0.021	2.80
gluten concentration	0.142	2	0.071	9.47**
EDC x threonine	0.282	4	0.070	9.40**
EDC x protein	0.049	4	0.012	1.60
error	0.090	12	0.0075	
total	0.647	26		

$$F_{(0.01)2, 12} = 6.93^{**}; F_{(0.05)2, 12} = 3.88^{*}$$

^aThe sum of squares (SS) values for pH, reaction time, reaction temperature, polarity and the interaction between threonine and gluten concentration were small and were incorporated into the error term.

Figure 6. Effect of gluten concentration on the attachment of threonine to gluten.

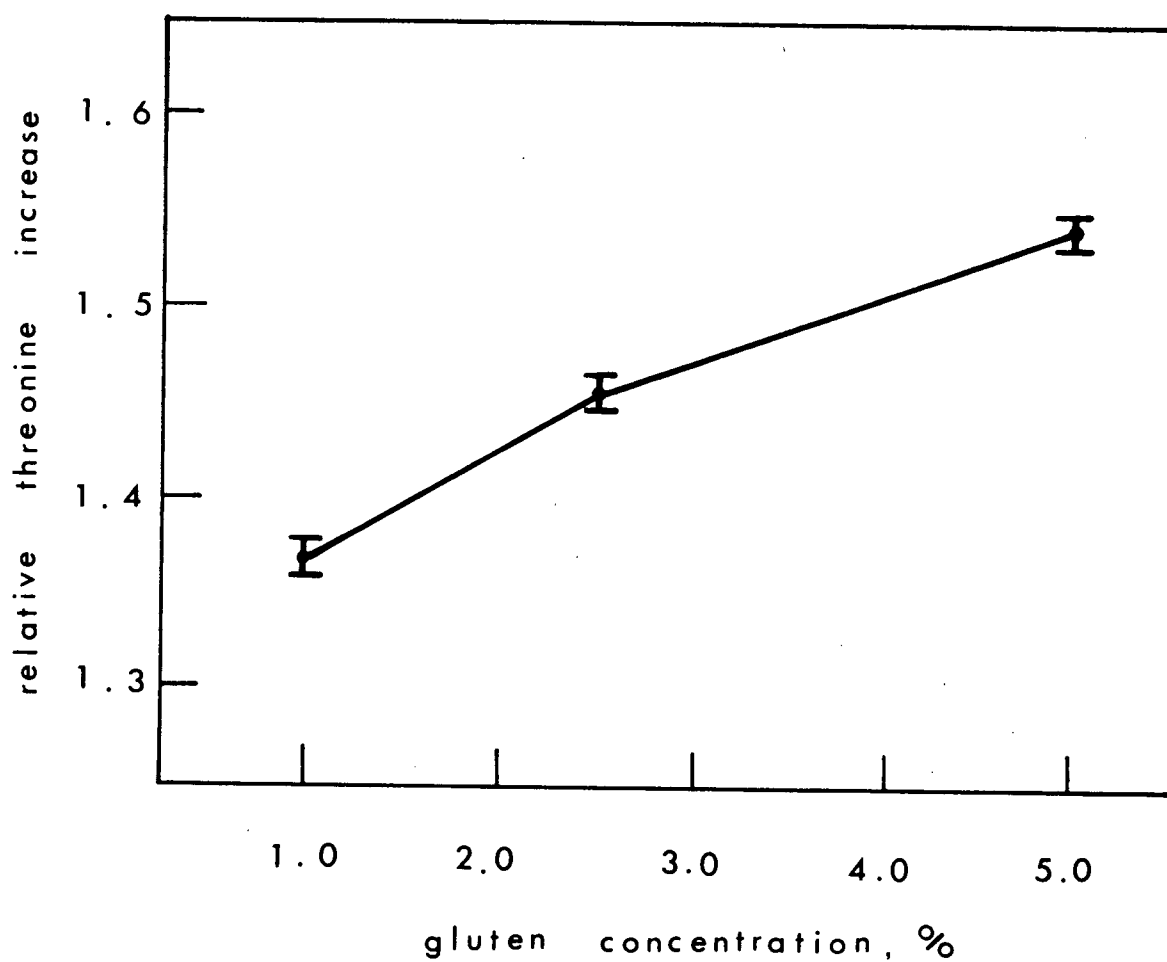
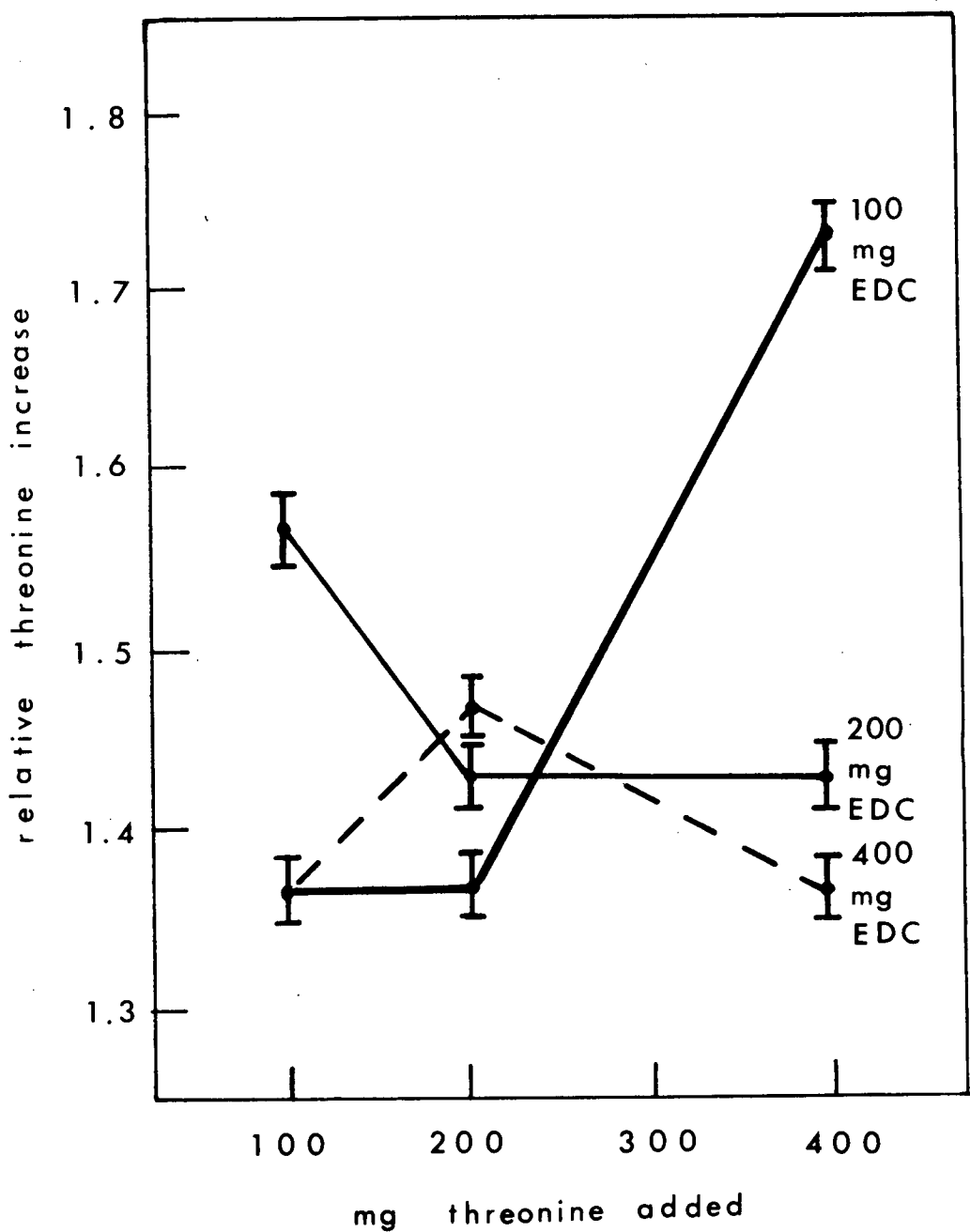


Figure 7. Effect of the interaction between threonine and EDC levels on the attachment of threonine to gluten.



produces a stable carboxylate derivative and releases the isourea, thereby shifting the equilibrium towards complete reaction of the carboxyl groups. The need for a high molar ratio of threonine to EDC, as well as the high concentration of gluten, may suggest the relative lability of the EDC-activated intermediates or poor nucleophilicity of threonine, and the requirement of high concentrations of available amino and carboxyl groups to encourage the formation of a stable peptide bond rather than the side reactions.

Relative threonine increases ranging from 1.2-fold to 1.8-fold were achieved with an average increase of 1.5-fold. Yields of the covalently threonine enriched gluten ranged from 92 to 96%. The reaction conditions leading to the greatest extent of attachment of threonine in these experiments were 5% gluten (250 mg/5 ml), 100 mg EDC, 400 mg threonine, pH 6.5 and reaction time of 4 hours at 22°C.

7. Simultaneous attachment of lysine, N^ε-acetyl lysine or N^ε-benzylidene lysine and threonine to gluten

In an effort to conserve reagent (EDC) and time for preparation of enriched products, preliminary experiments were conducted to investigate the feasibility of simultaneous attachment of lysine or lysine derivatives and threonine to gluten. Table 22 shows the results of these experiments.

When gluten was incubated with each amino acid or amino acid derivative individually during the carbodiimide reaction, relative lysine increases of 3.4, 1.9 and 2.0 were obtained using 1 millimole of N^ε-acetyl lysine, lysine.HCl and N^ε-benzylidene lysine, respectively, and relative threonine increases of 1.9 and 1.4 were obtained using 4 millimoles and 2

Table 22. Simultaneous reaction of lysine or lysine derivatives and threonine with gluten^a.

reactants	relative lysine increase	relative threonine increase
200 mg N ^ε -acetyl lysine 480 mg threonine	1.2	1.2
200 mg lysine.HCl 480 mg threonine	1.4	1.1
250 mg N ^ε -benzylidene lysine 480 mg threonine	1.4	1.1
200 mg N ^ε -acetyl lysine 240 mg threonine	1.9	1.1
200 mg lysine.HCl 240 mg threonine	1.6	1.1
250 mg N ^ε -benzylidene lysine 240 mg threonine	1.8	1.0
200 mg N ^ε -acetyl lysine	3.4	-
200 mg lysine. HCl	1.9	-
250 mg N ^ε -benzylidene lysine	2.0	-
480 mg threonine	-	1.9
240 mg threonine	-	1.4

^a Reaction conditions: 2.5% (500 mg/20 ml) gluten; 300 mg EDC; 50 mg NHS; amino acid levels as specified (the weights represent 1 millimole of lysine or its equivalent; 2 or 4 millimoles of threonine); pH 5.7; 40 °C for 6 hours.

millimoles of threonine, respectively. When lysine or lysine derivative was present simultaneously in the reaction mixture with threonine, the extent of attachment of both types of amino acids was drastically reduced. Threonine appears to be less reactive than lysine in the coupling reaction mediated by carbodiimide and almost no increase in covalent threonine content resulted during the reaction in the presence of lysine or its N^{ϵ} -protected derivatives, even when threonine was added in 4-fold higher molar concentration than lysine. On the other hand, the presence of threonine also inhibited the attachment of lysine and its derivatives to gluten. For example, relative lysine increase decreased from 3.4 for the control (N^{ϵ} -acetyl lysine only) to 1.9 (in the presence of 240 mg threonine) to 1.2 (in the presence of 480 mg threonine). Competition between the two types of amino acids for EDC or for activated carboxyl groups on gluten may have resulted in the destruction of the labile O-acylisourea intermediate before the production of a peptide bond. It is also possible that peptide bonds were formed, but linking the two types of amino acids to form dipeptides rather than attaching them onto gluten.

These results indicate that under these reaction conditions, the simultaneous presence of the two amino acids resulted in decreased efficiency of attachment for both lysine or its derivative and threonine.

PART II. PREPARATION OF COVALENTLY ENRICHED GLUTEN BY THE PLASTEIN REACTION

Enzymatic protein degradation and resynthesis for protein improvement was attempted using the plastein reaction. Gluten was first enzymatically hydrolyzed into small peptides by incubation with pepsin for 48 hours; the hydrolysate was subsequently incubated with papain for another 48 hours to induce plastein formation. To improve the content of the essential amino acids in the plastein produced, lysine ethyl ester or threonine was added to the incubation mixture with papain. It was observed that almost all these mixtures formed gels during the incubation. After dialysis and freeze-drying, the amino acid content of the products was analyzed. Table 23 shows the composition of the "plasteins" along with that of the starting material, the 48 hour pepsin hydrolysate of gluten.

Comparison of the amino acid composition of the plasteins to the starting material indicates large differences in the content of many of the amino acids, including pronounced reduction in glutamate (or glutamine) and proline, and increases in aspartate (or asparagine), threonine, valine, methionine, isoleucine, leucine, lysine and arginine. The addition of lysine ethyl ester into the incubation mixture resulted in higher levels of lysine content in the "lysine-plastein" compared to control plastein, whereas the addition of threonine only resulted in slightly higher levels of threonine in the "threonine-plastein."

Yield of the plasteins was very low, being 8 to 12% based on the starting weight of pepsin hydrolysate used. The poor yields

Table 23. Amino acid composition of control and enriched plasteins compared to the starting material, 48 hour pepsin hydrolysate of gluten^a.

	g/100 g					
	48 hr. hydrolysate	control plastein	lysine plastein		threonine plastein	
			1:8	1:2.4	1:8	1:2.4
asp	2.95	4.31	4.41	5.37	4.78	4.95
thr	2.26	2.99	2.77	3.67	3.09	3.54
ser	4.08	4.56	4.23	4.99	4.98	5.04
glu	39.51	20.61	23.71	19.20	20.11	23.05
pro	13.15	8.59	8.02	8.41	6.62	7.13
gly	2.94	3.02	2.56	3.21	3.27	3.04
ala	2.06	2.19	2.15	3.03	3.27	2.92
val	3.01	6.67	4.93	5.37	6.53	5.16
met	1.05	2.75	2.41	2.60	3.38	2.87
ile	3.43	9.19	9.05	8.30	8.72	8.21
leu	6.27	12.83	13.27	11.83	12.92	11.76
tyr	3.31	4.93	4.54	4.59	4.57	4.88
phe	4.65	4.57	4.57	4.38	3.31	4.02
lys	1.25	2.45	3.14	4.63	3.35	2.99
his	1.99	1.87	1.95	1.96	2.25	1.97
arg	3.09	3.46	3.28	3.44	3.86	2.67

^aPlasteins were prepared by incubation of 48 hour pepsin hydrolysates of gluten (35% w/v) concentration with papain (1% w/w) at pH 6.0 for 48 hours at 37 °C, in aqueous medium containing 0.01M cysteine and 20% acetone. "Lysine plastein" and "threonine plastein" contained, in addition to the above, either lysine.OEt.diHCl or threonine in ratios of 1:8 or 1:2.4 (w/w, amino acid: gluten) during incubation.

^bGluten (1.2%, w/v) was incubated with pepsin (1%, w/w) for 48 hours at 35 °C, pH 1.6. The pH of the hydrolyzed gluten was then adjusted to 6.0, and after centrifugation at 3000 x g for 15 minutes, the supernatant was freeze-dried to yield the starting material for the plastein reaction.

suggest that many small peptides and free amino acids were formed during pepsin hydrolysis, and that subsequent incubation with papain was not effective in significantly increasing the molecular weight of these hydrolysate peptides, which would therefore be lost during dialysis. The inability to detect the formation of high molecular weight protein-like material has been reported by v. Hofsten and Lalasidis (1976) and Edwards and Shipe (1978). Although insoluble matter which was often gel-like resulted from incubation with papain, it is possible that physical, non-covalent forces of attraction are responsible.

The lysine and threonine contents of the nondialyzable fraction of the product are higher than the starting material. However, whether or not this is a result of plastein formation, i.e. re-synthesis of peptide bonds, is not clear. These high levels may be due to selective hydrolysis by pepsin of other amino acids, leaving relatively intact a portion of gluten which is of high molecular weight and has high content of the amino acids lysine and threonine. Addition of lysine ethyl ester or threonine to the incubation mixture did not consistently ensure higher levels of the amino acid in the resulting plastein.

Further experiments were conducted to investigate feasibility of using gluten or acid-solubilized gluten for enzymatic incorporation of lysine or threonine. The amino acid compositions of these plasteins and their starting materials are shown in Tables 24 and 25. In analogy to the experiments utilizing pepsin-hydrolyzed gluten as substrate, these plasteins also showed large dissimilarity in their amino acid content compared to the starting materials. Lysine and threonine contents were high in the control plasteins, and were not consistently improved

Table 24. Amino acid composition of control and enriched plasteins compared to the starting material, gluten^a.

	g/100 g					
	gluten	control plastein	lysine plastein		threonine plastein	
			1:8	1:2.4	1:8	1:2.4
asp	2.81	6.52	6.44	6.47	6.90	6.72
thr	2.23	3.60	3.40	3.63	3.79	3.66
ser	4.48	5.05	4.74	4.88	4.97	4.77
glu	37.45	25.32	24.97	24.54	24.23	21.97
pro	12.67	7.24	6.97	6.14	6.84	6.05
gly	2.88	3.16	3.04	3.16	3.28	3.35
ala	2.17	4.00	4.02	4.07	4.30	4.31
val	2.96	4.29	4.89	4.50	4.37	4.54
met	1.16	2.59	3.10	2.85	2.73	2.81
ile	3.15	3.66	3.31	4.47	4.54	3.36
leu	6.50	9.01	9.48	8.85	9.19	9.32
tyr	3.09	4.31	4.21	4.34	4.45	4.38
phe	4.86	4.03	3.76	4.03	3.53	3.72
lys	1.33	4.75	4.46	4.56	4.46	4.96
his	1.78	2.42	2.88	2.93	2.68	3.07
arg	3.00	5.02	5.31	5.56	5.71	5.99

^aThe plasteins were prepared as described in the footnote to Table 23, except that gluten was used as the starting material.

Table 25. Amino acid composition of control and enriched plasteins compared to the starting material, 0.05N HCl solubilized gluten^a.

	g/100 g					
	acid gluten	control plastein	lysine plastein		threonine plastein	
			1:8	1:2.4	1:8	1:2.4
asp	2.58	3.42	4.41	4.18	4.32	4.88
thr	2.42	2.79	3.01	2.96	3.06	3.19
ser	4.34	4.69	4.85	4.64	4.89	4.89
glu	38.21	34.98	31.34	31.23	31.33	27.70
pro	13.40	10.07	8.06	8.16	8.64	7.31
gly	2.96	2.58	2.32	2.36	2.76	2.60
ala	2.25	3.72	3.41	3.29	3.87	3.90
val	3.08	3.41	3.95	3.94	4.51	4.54
met	1.29	2.08	2.75	2.47	2.89	3.11
ile	3.10	3.75	3.69	4.63	3.61	5.00
leu	6.67	7.57	9.74	8.90	9.23	9.62
tyr	3.13	3.42	3.77	3.90	3.66	4.09
phe	5.44	4.11	3.71	3.71	3.55	3.31
lys	1.25	2.09	3.04	3.63	2.68	3.40
his	1.75	2.13	2.53	2.57	2.56	2.52
arg	3.13	4.18	4.41	4.42	3.41	4.91

^aPlasteins were prepared as described in the footnote to Table 23, except that gluten solubilized by acid treatment (0.05N HCl, 121 °C, 15 minutes) was used as the starting material.

further by the presence of added amino acids in the incubation mixture.

Table 26 summarizes the lysine and threonine contents in the various products and also compares the yields of product. Even when untreated vital gluten was used as the starting material, the yield of product after incubation with papain was low (36%). Peptide bond hydrolysis or transpeptidation rather than condensation could explain these results. Similar trends were apparent using acid-solubilized gluten. The dialyzer tubing used in the present study (Fisher 8-677B) is highly permeable to water and low molecular weight compounds, retaining only materials with molecular weight of 12,000 or greater. The higher plastein yields obtained by Fujimaki et al. (1977) may have been due to the use of ultrafiltration techniques for retention of products possessing molecular weight of 500 or greater. In some cases, the plastein yield reported by these workers was based on the starting weight of the dialyzable portion of pepsin hydrolysates of the protein, rather than on the basis of the starting weight of the original protein. For example Aso et al. (1974) obtained a dialyzable fraction with 65% yield, and upon using this as the substrate, obtained plastein (TCA-insoluble) products in 61.9% yield, approximately half of which were water-insoluble. The formation of the water-insoluble products was postulated to be due to hydrophobic assembly of the chains in water.

Table 26. Comparison of product yields, lysine and threonine contents in "plasteins" formed from various substrates.

substrates	%	g/100 g	
		lysine	threonine
		(1.3) ^a	(2.2) ^a
gluten	36	4.8	3.6
lysine + gluten (1:8)	38	4.5	3.4
lysine + gluten (1: 2.4)	40	4.6	3.6
threonine + gluten (1:8)	37	4.5	3.8
threonine + gluten (1:2.4)	36	5.0	3.7
acid gluten	51	2.1	2.8
lysine + acid gluten (1:8)	37	3.1	3.0
lysine + acid gluten (1:2.4)	39	3.6	3.0
threonine + acid gluten (1:8)	32	2.7	3.1
threonine + acid gluten (1:2.4)	31	3.4	3.2
pepsin gluten	12	2.5	3.0
lysine + pepsin gluten (1:8)	9	3.1	2.8
lysine + pepsin gluten (1:2.4)	8	4.6	3.7
threonine + pepsin gluten (1:8)	11	3.4	3.1
threonine + pepsin gluten(1:2.4)	11	3.0	3.5

^aLysine and threonine contents in wheat gluten (untreated)

PART III. EVALUATION OF NUTRITIONAL QUALITY OF COVALENTLY ENRICHED GLUTEN

1. In vitro evaluation (DNBS-availability and pepsin-pancreatin-digestibility)

Amino acid analysis after acid hydrolysis allows determination of the "total" amino acid composition of a protein. However, it cannot indicate the nutritional availability of the amino acids. The DNBS reaction was therefore used to measure the content of "free" ϵ -amino groups or "available" lysine in the lysine-linked gluten samples, while an in vitro pepsin pancreatin digestion test was carried out to indicate the relative ease of enzymatic hydrolysis of the attached lysine residues. These values were measured on various gluten samples enriched by carbodiimide mediated covalent coupling of lysine or lysine derivatives. The results were compared with the total lysine content measured after p-toluene-sulfonic acid hydrolysis, as shown in Table 27.

The percentage of lysine residues in lysine-linked acid gluten measured by the DNBS reaction was low (48%), implying that the ϵ -amino groups of the lysine residues may have been blocked, perhaps in the form of α - ϵ or γ - ϵ bonds as a result of condensation with α -(C-terminal amino acid) or γ -(glutamic acid residue) carboxyl group of acid-solubilized gluten or even with α -carboxyl group of the added lysine. In contrast, in the case of acid-solubilized gluten reacted with N ϵ -benzylidene lysine, followed by removal of the benzylidene protecting group, the proportion of DNBS-available lysine ϵ -amino groups was high (91%), indicating the effectiveness of the protecting group during the carbodiimide reaction and of the unblocking procedure after the reaction. Pepsin pancreatin digestion

Table 27. Comparison of lysine contents measured after acid hydrolysis, dinitrobenzenesulfonate (DNBS) reaction and in vitro pepsin pancreatin digestion (PPD).

samples	lysine (g/100 g) ^a		
	acid	DNBS	PPD
gluten	1.4	1.2 (89)	1.0 (75)
0.5N HCl gluten	1.5	1.3 (87)	0.6 (41)
lysine acid gluten	5.5	2.6 (48)	2.3 (41)
N ^ε -benz. lysine acid gluten ^b	3.1	2.8 (91)	1.3 (41)
pepsin (38 hr) gluten	1.5	d	1.0 (69)
N ^ε -benz. lysine pepsin (38 hr) gluten ^b	5.9	5.2 (89)	3.8 (65)
pepsin (1 hr) gluten	1.4	d	1.0 (75)
N ^ε -benz. lysine pepsin (1 hr) gluten ^b	5.9	5.3 (90)	3.9 (65)
lysine gluten	2.5	1.9 (76)	1.3 (52)
N ^ε -acetyl lysine gluten ^c	4.4	1.4 (32)	1.2 (27)
N ^ε -benz. lysine gluten ^c	2.6	2.1 (81)	1.5 (58)
egg (whole)	6.2	5.4 (87)	1.9 (31)
casein (isoelectric)	7.3	7.3 (100)	3.3 (45)

^a Lysine contents measured by DNBS and PPD were corrected for nitrogen content of each sample. Numbers in parentheses indicate the content of DNBS-available and PP-digestible lysine as a percentage of the total lysine content measured after acid hydrolysis.

^b N^ε-benzylidene group was removed after the carbodiimide reaction, prior to lysine determination.

^c N^ε-acetyl and N^ε-benzylidene groups were not removed after the carbodiimide reaction.

^d Not determined.

showed lower percentages (41%) of pepsin-pancreatin-digestible lysine for the acid-solubilized gluten as well as the corresponding lysine and N^ϵ -benzylidene lysine enriched samples, when compared to the value obtained for untreated gluten. This may have been due to the presence of $\gamma - \alpha$ bonds or $\beta - \alpha$ bonds (β -carboxyl group of aspartic acid residue). Alternatively, it may reflect a reduction in overall digestibility by these two enzymes after acid solubilization. When compared to untreated gluten, the acid-solubilized gluten samples showed decreased enzymatic release of several of the amino acids, especially aspartic acid, threonine and serine (Table 28). However, the pepsin-pancreatin-digestible lysine content in lysine acid gluten is still comparable to the content in whole egg (Table 27).

In contrast to the acid-solubilized samples, the lysine enriched samples prepared from N^ϵ -benzylidene lysine reaction with pepsin-hydrolyzed gluten, followed by acid cleavage of the benzylidene group, showed high proportions of both DNBS-available and pepsin-pancreatin-digestible lysine. The digestion profiles showed similar release of amino acids to that from untreated gluten, with the exception of slightly lower values of isoleucine, which may be explained by the lower total content of this amino acid after pepsin hydrolysis of gluten.

The lysine gluten sample showed somewhat low percentages of both DNBS-available (76%) and pepsin-pancreatin-digestible (52%) lysine contents. This suggests that some isopeptide bonds linking the ϵ -amino group are involved, but some of the lysine residues are attached by peptide bonds, leaving the ϵ -amino group free, since the actual DNBS-available lysine content in the lysine gluten is higher than the total lysine content.

Table 28. Profile of amino acids released by pepsin pancreatin digestion.

	g released/100 g protein sample					
	gluten	N ^ε -benz.lys. 38 hr pepsin gluten	acid gluten	N ^ε -benz.lys. acid gluten	egg	casein
asp	0.3	0.3	0.1	0.1	1.2	0.5
thr	2.3	1.9	0.6	0.6	1.1	1.0
ser	0.6	0.7	0.3	0.3	0.9	0.5
glu	0.4	0.4	0.5	0.4	1.1	0.8
gly	0.1	0.1	0.1	0.1	0.3	0.2
ala	1.3	1.3	1.0	1.0	1.3	0.9
met	0.8	0.7	0.6	0.6	1.5	1.0
ile	1.1	0.8	1.4	1.4	0.9	0.6
leu	4.2	2.8	4.0	3.8	5.2	4.5
tyr	1.7	1.8	1.5	1.3	2.6	2.0
phe	2.4	2.4	2.0	2.0	3.0	3.0
lys	1.0	3.8	0.6	1.3	1.9	3.3
his	0.6	0.5	0.4	0.5	0.7	0.6
arg	2.4	2.2	1.9	2.0	2.0	2.5
total ^a	19.8	20.2	15.7	16.0	24.9	20.9
EAA ^b	15.4	16.0	12.1	12.5	18.2	16.9

^aContents of proline, cysteine and tryptophan were not included in the total (g released/100g) content.

^bContents of cysteine and tryptophan were not included in the content of essential amino acid (EAA) released/ 100 g protein sample.

in the gluten control.

N^ε-Acetyl lysine gluten showed virtually no increase in either DNBS-available or pepsin-pancreatin-digestible lysine content when compared to the control. Since the N^ε-acetyl groups were not intentionally removed after the carbodiimide reaction, they would still protect the lysine ε-amino groups and render them inaccessible to the DNBS reagent. The low pepsin-pancreatin-digestible lysine content may be deceiving. In fact, the N^ε-acetyl lysine residues may have been readily released by enzymatic digestion. However, since N^ε-acetyl lysine emerged at the same position as proline in the amino acid analysis ion exchange elution profile, it was not possible to resolve the two peaks to give a reliable estimate of either amino acid.

In contrast to N^ε-acetyl lysine gluten, the N^ε-benzylidene lysine gluten sample showed high DNBS-available content(81%) and fairly high pepsin-pancreatin-digestible content (58%). Although the benzylidene group was not intentionally removed in this case, this group is very sensitive to acidic conditions, being a Schiff's base type of linkage (Bodanszky et al., 1976). It is possible that partial removal of the protecting group occurred during addition of acetic acid to stop the carbodiimide reaction and during subsequent dialysis against 1N acetic acid.

It should be noted that hydrolysis by the pepsin pancreatin digestion assay is not complete (Stahmann and Woldegiorgis, 1975). As shown in Table 27, 31% and 45% of the total lysine were released under the conditions of this assay for whole egg and isoelectric casein, respectively. Gluten showed 75% release of its lysine. Comparable values were obtained for the total amino acids released by the three proteins,

although egg had generally higher contents of released amino acids than gluten or casein (Table 28).

Analysis of the total amino acid compositions indicates that the ratio of essential amino acids to total amino acids (E/T) is lower for wheat products than for whole egg, the reference protein. This is not necessarily bad, since egg has an E/T ratio almost twice as high as required for most efficient use of its essential amino acids (Pomeranz, 1971). A pepsin-pancreatin digestion (PPD) index has been suggested which combines the pattern of essential amino acids released by in vitro digestion to the pattern of the residues remaining after the digestion (Akeson and Stahmann, 1964). The PPD index is calculated in a similar manner to the calculation of the pepsin digest residue (PDR) index described by Sheffner et al. (1956) for samples digested in vitro by pepsin. These indices have been claimed to be good predictors of biological value when digestibility coefficients of samples are taken into consideration.

PPD indices were calculated for samples described in Table 27, using the amino acids lysine, phenylalanine + tyrosine, methionine, threonine, valine, isoleucine, leucine and histidine. Cystine was not included in the calculation because it was liberated in trace quantities and it had been reported that the PPD index values were not significantly changed when cystine was omitted (Stahmann and Woldegiorgis, 1975). Tryptophan was also excluded as it had been reported that the correlation between PPD index and biological value was the same whether tryptophan was included or excluded (Akeson and Stahmann, 1964). Arginine, which has been classified as a semi-indispensable amino acid and is required only

in small amounts, was not included in the calculation since better correlation with biological value was obtained when PPD index was calculated without arginine (Akeson and Stahmann, 1964).

The PPD indices were calculated to be 100 for whole egg; 84 for casein and 27 for gluten. These values rank the three proteins in the same order as has been reported by Akeson and Stahmann (1964), who obtained the following values for whole egg, casein and gluten, respectively: 100, 78 and 49 for PPD index values; 100, 66 and 40 for chemical score values; and 100, 89 and 55 for essential amino acid index values. Covalently enriched gluten samples had higher PPD indices than gluten controls. The calculated values were 27, 33 and 46 for gluten, lysine gluten and N^ε-benzylidene lysine pepsin (38 hour) gluten, respectively, and 44 and 51 for acid gluten and N^ε-benzylidene lysine acid gluten, respectively. However, the enriched glutens had lower PPD indices than whole egg or casein. The ratio of essential amino acids released/total amino acids released was comparable for all samples (Table 28), but the profile of essential amino acids released differed between different protein samples. Lysine was not the limiting amino acid in the N^ε-benzylidene lysine pepsin gluten, since the amount of lysine released was even greater than that released in the egg or casein sample. However, this sample had low values of methionine, and tyrosine + phenylalanine, which may have contributed to the low PPD index. The acid gluten control had low values for both total and essential amino acids released; although the released lysine content was increased in the N^ε-benzylidene lysine acid gluten sample, a number of the other essential amino acids were still released at levels much lower than the egg or casein

sample, and the PPD index was low.

Recent reports on in vitro digestion tests as indicators of protein quality have criticized the current methods of calculation of indices such as the PPD index (Marable and Sanzone, 1980). Instead recommendations have been made to use an exponential-type relationship between protein quality and a limiting rate of digestion for one amino acid or between protein quality and the product of a limiting rate of digestion times an essential amino acid composition function. Until the testing of these relationships are completed, conclusions that can be derived from in vitro enzymatic hydrolysis tests may be limited.

Despite the lack of complete hydrolysis by this method, the amount of the limiting amino acid released by enzymatic hydrolysis is a good indicator of amino acid availability (Stahmann and Woldegiorgis, 1975). The high values of pepsin-pancreatin-digestible lysine for the pepsin-hydrolyzed gluten samples enriched using N^ϵ -benzylidene lysine are comparable to or higher than the values for the whole egg and isoelectric casein standards. These results indicate potential use of these high lysine gluten samples as good quality proteins.

These preliminary tests thus demonstrate good digestibility and availability of the lysine-enriched gluten samples, particularly those samples in which α - α peptide bonds to attach the lysine were encouraged. It is possible that isopeptide links and N^ϵ -substituents of lysine, which were not measured by these in vitro analyses, may be nutritionally available in the body. For example, ϵ -(γ -glutamyl)-L-lysine and ϵ -(α -glutamyl)-L-lysine have both been found to be available as lysine sources for the rat (Mauron, 1972; Carpenter, 1973).

N^ϵ -Benzylidene lysine has also been shown to be virtually 100% utilized as a lysine source during growth trials on rats (Finot et al., 1977; Finot et al., 1978). N^ϵ -Acetyl lysine is also probably biologically available. One of the pathways of lysine degradation in mammalian tissue and in organisms such as yeast, proceeds through acylated intermediates; N^ϵ -acetyl lysine is formed in the liver and the acyl group is later removed (White et al., 1973). Finot et al. (1978) tested various N-substituted lysine derivatives for their growth-promoting effect in rat assays. ϵ -N-(α -L-aminoacyl), α -N- ϵ -N-di-L-aminoacyl and Schiff base type derivatives were all utilized efficiently as lysine sources for the rat. None of the α -N-acyl- and ϵ -N-acyl-glycyl derivatives were utilized at all. ϵ -N-Acyl and ϵ -N-(ω -L-aminoacyl) derivatives were generally not utilized, with the exception of ϵ -N-(γ -L-glutamyl)lysine and ϵ -N-formyl- and ϵ -N-acetyl lysine. Kornguth et al. (1963) observed that papain, chymotrypsin, trypsin, leucine amino peptidase and Pronase did not attack the ϵ -(γ -glutamyl) lysine. However, this dipeptide is probably absorbed into the intestinal wall and hydrolyzed in the kidney since it can be found in the plasma of rats and chicks fed it in their diet (Waibel and Carpenter, 1972), and an ϵ -lysine acylase has been suggested to function in the mammalian kidney (Leclerc and Benoiton, 1968). Although poly- ϵ -N-methyl, ϵ -N-dimethyl-L-lysine was resistant to hydrolysis by trypsin, it was hydrolyzed by porcine carboxypeptidase B (Seely and Benoiton, 1969), demonstrating that substitution on the side chain amino group does not necessarily hinder proteolysis at the main chain peptide backbone.

2. Microbiological evaluation (Tetrahymena bioassay)

The use of the protozoan Tetrahymena pyriformis W for protein quality evaluation relies on its mammalian-like requirement for essential amino acids and the possession of enzyme systems somewhat comparable to those found in higher animals (Kidder and Dewey, 1951). However, although Tetrahymena can utilize intact proteins, pre-digestion of protein samples prior to the assay is required to obtain results which correlate well with other assays such as the rat PER assay (Smith and Pena, 1977; Landers, 1975; Evans and Witty, 1980). Preliminary experiments were carried out to compare the pre-digestion of casein, gluten and enriched gluten samples with pepsin at pH 1.6, according to Evancho et al. (1977) and with papain at pH 7.2, according to Evans et al. (1978).

The results of Tetrahymena growth on four different samples subjected to pepsin and papain pre-digestion are shown in Table 29. Pepsin pre-digestion in general gave higher organism counts/ml than papain pre-digestion. Standard deviation values were also lower in the former case. It was observed that gluten samples were not well dispersed after papain digestion, and the difficulty in obtaining typical aliquots of sample for the assay may have contributed to the large coefficient of variation. The measurement of growth without prior enzymatic hydrolysis has been suggested to relate more closely to protein solubility than to protein quality (Evans and Witty, 1980). Although casein was more effectively solubilized by papain than by pepsin pre-digestion, it was reported that in the case of ANRC casein, pre-digestion was not a critical factor, since suspensions of casein were utilized for organism growth as well as their pepsin digests (Landers, 1975).

Table 29. Comparison of pepsin versus papain for pre-digestion of casein and gluten samples for Tetrahymena assay.

	pepsin pre-digestion		papain pre-digestion	
	10^4 org/ml	mean 10^4 org/ml ^a	10^4 org/ml	mean 10^4 org/ml ^a
casein	83, 88, 87	86 ± 3 (3%)	75, 79, 78	77 ± 2 (3%)
gluten	44, 38, 42	41 ± 3 (7%)	19, 23, 32	25 ± 7 (28%)
gluten + 1%lys	78, 81, 86	82 ± 4 (5%)	85, 90, 78	84 ± 6 (7%)
1%lys-gluten	75, 83, 84	81 ± 5 (6%)	70, 65, 80	72 ± 8 (11%)

^aStandard deviation was calculated as $\pm \left(\sqrt{\frac{\sum(y - \mu)^2}{n - 1}} \right)$, where y = individual count/ml, μ = average count/ml and n = number of replicates used to calculate the average. Numbers in parentheses represent the coefficients of variation, calculated as 100x(standard deviation divided by the mean)

Pepsin digestion has the advantage that microbial growth is minimized during the digestion period since samples are at pH 1.6. However, the increase of ionic strength in digests due to the acidification and subsequent neutralization prior to the assay may affect organism growth. Frank et al. (1975) indicated that ionic imbalance may adversely affect Tetrahymena, whereas Voight et al. (1979) and Evans et al. (1978b) found that moderate salt concentrations had little effect. In the case of gluten, the low pH at which digestion is carried out for pepsin hydrolysis may be advantageous due to the greater solubility of this protein at low pH values. At the neutral pH for papain digestion, gluten forms an insoluble mass which may render peptide bonds inaccessible for proteolysis. Contamination and multiplication of undesirable microorganisms are also more likely under neutral pH conditions.

Based on the better solubilization of gluten samples and lower values for coefficient of variation when using pepsin pre-digestion, subsequent assays were conducted on protein samples after pepsin pre-digestion.

Evaluation of protein quality using Tetrahymena pyriformis W bioassay is based on the calculation of relative nutritive value or RNV, which is the ratio of organism count in the test sample medium to organism count in a reference protein (ANRC casein), multiplied by 100. Since various workers using this bioassay have reported considerable variability and requirement for strict control over growth conditions in order to obtain meaningful results, the repeatability of the results within an assay as well as reproducibility between assays carried out on separate occasions were first assessed by calculating coefficients of variation for Tetrahymena

grown on the standard protein, ANRC casein.

Table 30 shows the organism counts/ml for duplicates in five assays. Organisms from each flask were counted at least twice on a haemocytometer. The average organism count ranged from 61 to 104×10^4 organisms/ml for the five assays, with coefficients of variation ranging from 2% to 8%. An average organism count based on the ten values was calculated to be $86 \pm 15 \times 10^4$ organisms/ml. This represents a coefficient of variation of 17%; this large value may have been contributed by the low counts obtained in assay number 3. During the incubation of these samples, temperature accidentally dropped from 27°C to 22°C. Cells in the exponential growth phase of the life cycle are strongly temperature dependent, and the temperature at which maximum growth is usually achieved is about 29°C (Hill, 1972). If the values from assay number 3 were omitted from the calculation, an average organism count based on the eight values was $92 \pm 9 \times 10^4$ organisms/ml. The coefficient of variation of 10% is comparable to that reported by Evancho *et al.* (1977) and Srinivas *et al.* (1975) using direct microscopic counting to evaluate organism growth. Warren and Labuza (1977) reported a standard deviation for the casein control of $\pm 18.6\%$ RNV. The results reported in Table 30 confirm the precautionary warning given by most workers in the field, that considerable variability can result due to inherent biological variability of the organisms, fluctuations in growth conditions and error due to the counting techniques. In spite of the large coefficient of variation between assays carried out on separate days, good repeatability between duplicates within an assay was achieved. It is concluded that the reference protein should be included in each assay in order that

Table 30. Standard deviation for growth of Tetrahymena using ANRC casein as a protein source.

assay number	organism count (10^4 /ml)	average count (mean) within assay (10^4 /ml) ^a
1	81, 87	84 ± 4 (5%)
2	92, 89	90 ± 2 (2%)
3	58, 64	61 ± 4 (6%)
4	93, 83	88 ± 7 (8%)
5	108, 101	104 ± 5 (5%)

^aStandard deviation was calculated as described in the footnote to Table 29. Numbers in parentheses represent coefficients of variation.

relative nutritive values obtained from different assays can be comparable.

Evancho et al. (1977) and Warren and Labuza (1977) attributed much of the error in the bioassay to the use of direct microscopic count for growth determination. In the present study, the TPTZ dye reduction colorimetric test was investigated as an alternative method for evaluation of Tetrahymena growth. In general, both methods ranked test proteins in the same order of protein quality. However, since the dye reduction test requires rather high concentrations of organism for accurate detection (at least 10^5 organisms/ml), the sensitivity of this test was limited particularly when evaluating gluten and baked gluten samples, in which growth of organisms was poor. The dye reduction test was also considered to be relatively nonspecific, due to its inability to detect differences in cell size of Tetrahymena grown on different protein sources, and also its inability to distinguish between reduction of the dye by Tetrahymena from reduction by microbial contaminants. Inadvertent introduction of contaminants occasionally led to false high readings. Possible interference by components of the test sample was corrected by including blanks consisting of autoclaved aliquots of sample suspension. Since the dye reduction test is based on the number of living organisms after precisely 96 hours of incubation, samples had to be assayed without delay. Although this test is theoretically capable of assaying many samples at a time, in practice, this was limited by the need to centrifuge many samples simultaneously. In contrast, although the direct microscopic counting technique was tedious, flexibility in time and opportunity to re-count samples when desired were important advantages since samples were maintained in preserving fluid. Consideration of these factors led

to the choice of the direct microscopic counting technique for determination of Tetrahymena growth .

Table 31 shows the average RNV for gluten or acid gluten samples and corresponding samples enriched by covalent attachment or free addition of N^ε-acetyl lysine, N^ε-benzylidene lysine or lysine. All enriched samples had RNV approximating that of the standard casein, which was assigned a value of 100. Only the unsupplemented samples, gluten and acid gluten, had lower RNV of 54 and 64, respectively. The higher RNV of acid gluten may be due to better solubilization of this sample compared to gluten.

Growth of Tetrahymena on casein, wheat flour, wheat flour + L- lysine and wheat flour + L-lysine + DL-methionine + L-threonine were 70.6, 28.0, 66.7 and 81.0 x 10⁴/ml, respectively, according to Srinivas et al. (1975). These values represent RNV of 100, 40, 94 and 115, respectively. Kaestner et al. (1976) obtained RNV of 92.6 and 54.3 for casein and wheat, respectively, using logarithmic conversions of direct microscopic counts. The values reported by these workers are comparable to the values reported in Table 31.

Evancho et al. (1977) obtained a regression equation relating Tetrahymena RNV to rat PER values. This equation was used to calculate PER values, as shown in Table 31. The reference casein had a calculated PER of 2.5; enriched samples had calculated PER values ranging from 2.3 to 2.8, while gluten and acid gluten samples had calculated PER values of 1.5 and 1.7, respectively. The PER values obtained using male rats were reported as 2.3 and 1.5 for casein and wheat flour, respectively

Table 31. Average RNV and calculated PER values for gluten and enriched glutes using the Tetrahymena assay

	number of replicates	average RNV ^a	calculated PER ^b
casein	- ^c	100	2.5
gluten	9	54 ± 6	1.5
1% N ^ε -acetyl lysine gluten	8	98 ± 5	2.4
1% N ^ε -benz. lysine gluten	8	93 ± 13	2.3
1% lysine gluten	8	92 ± 12	2.3
gluten + 1% N ^ε -acetyl lysine	6	103 ± 17	2.5
gluten + 1% N ^ε -benz.lysine	6	95 ± 14	2.4
gluten + 1% lysine	6	93 ± 14	2.3
acid gluten (0.05N HCl)	4	64 ± 5	1.7
4% lysine acid gluten	4	116 ± 9	2.8
1% lysine acid gluten	4	100 ± 8	2.5

^a RNV = 100 x (organism count for sample/ organism count for casein)

^b calculated PER = 0.286 + (0.022)(RNV) (Evancho et al., 1977)

^c casein standard, in duplicate or triplicate, was used in each assay.

(Srinivas et al., 1975). Wheat gluten has somewhat lower protein nutritional quality than the total proteins from wheat flour (Pomeranz, 1971), but the general agreement between the PER results in Table 31 and those reported for wheat flour by Srinivas et al. (1975) suggest that the values obtained by the Tetrahymena assay correlate quite well with PER values by rat bioassay.

As Table 31 shows, the bioassay was unable to detect any differences in Tetrahymena multiplication between samples which were covalently enriched or enriched by free addition. It also did not differentiate between lysine, N^ε-acetyl lysine and N^ε-benzylidene lysine as sources of lysine for supplementation, indicating that the N^ε-substituted derivatives are utilized effectively. Not indicated in the results, however, are the differences in organism size between cultures grown on casein and those grown on gluten samples. The size of organisms grown on gluten samples (both enriched and control samples) was usually one-half to one-third that of organisms grown on casein. The size of Tetrahymena cells is commonly 50 x 30 μ, but two-fold variations from this are common (Hill, 1972), the size being highly dependent on factors including nutrient composition of medium, temperature of incubation, phase of growth, osmolarity of medium, etc. Kaestner et al. (1976) reported that cells grown in casein were usually large, turgid and oblong, while cells grown in bread crumbs were small and flat. Evancho et al. (1977) also suggested that cell size varies with good and poor protein quality, even at a particular nitrogen concentration. It is possible that even with addition of lysine, gluten is still a poorer source of protein for cell maintenance than casein, although it may be equivalent as a protein

source for cell growth or multiplication. The sensitivity of the organism to many environmental factors and nutrient composition suggest also the probability of as yet unidentified growth inhibitors in gluten or growth stimulants in casein, which may account for the difference in cell size. The presence of larger quantities of non-proteinaceous material in gluten (protein content of approximately 75%) compared to ANRC casein (protein content of approximately 95%) may suggest possible interference by the carbohydrate and lipid fractions of gluten. Some fatty acids have been demonstrated to inhibit Tetrahymena growth (Landers, 1975) and high fat concentrations can reduce availability of air for growth by creating a barrier between the aqueous medium and air (Evans and Witty, 1980). Carbohydrate concentrations exceeding 1.5% of the media were reported also to reduce growth (Evans et al., 1979). In contrast to the higher levels of lipid and carbohydrate in gluten, a typical analysis of ANRC casein reveals 1.5% ether extractables (by Mojonnier method) and 0.25% carbohydrate (as lactose); trace amounts of vitamins and minerals are present (Humko Sheffield, 1978).

Due to the sensitivity of Tetrahymena to various growth factors and conditions, the ability to detect small differences in quality are limited, especially when one is interested in differences contributing to cell maintenance as well as cell growth. However, the assay remains useful as a screening test to rank quality of proteins which are grossly different in quality (Evancho et al., 1977; Warren and Labuza, 1977; Kaestner et al., 1976). Based on this bioassay, gluten samples which were covalently enriched with lysine or with N^E-substituted acetyl and benzylidene derivatives were equivalent in protein quality for supporting

Tetrahymena growth, when compared to the reference casein protein.

Gluten samples enriched either by covalent attachment or by simple addition were found to be superior to the gluten control in protein quality.

PART IV COMPARISON OF BROWNING IN WHEAT GLUTENS ENRICHED BY COVALENT ATTACHMENT OR BY SIMPLE ADDITION

The production of color and flavor compounds by the Maillard browning reaction plays an important role in the aesthetic appeal and acceptability of bakery products such as bread. However, careful control is mandatory to prevent excessive browning, which could severely impair not only acceptability, but also nutritional quality. Moreover, some of the products of the Maillard reaction have been implicated in hepatic disorders, allergic responses, hyperexcitability and other abnormal symptoms (Adrian, 1974; Ambrose et al., 1961).

The addition of free lysine to food has the disadvantage, from a technological point of view, of the high reactivity of lysine with reducing sugars in the Maillard reaction (Underwood et al., 1959; Finot et al., 1978). This study was carried out to compare the extent of browning and destruction of lysine in baked gluten samples fortified by either covalent attachment or by free addition. The effect of using N^ε-substituted lysine derivatives for fortification was also investigated. Relative nutritive values of the baked samples were compared using a bioassay with Tetrahymena.

1. Comparison of crust portions

Table 32 shows the total lysine content in crusts of gluten samples before and after baking. For the control gluten sample, baking in the absence of glucose caused relatively little destruction of the protein-bound lysine. In the presence of 10% glucose, however, over 30% of the

Table 32. Loss of lysine in crust portions of enriched glutens
(0% or 10% glucose added) after baking at 190 °C, 10 minutes.

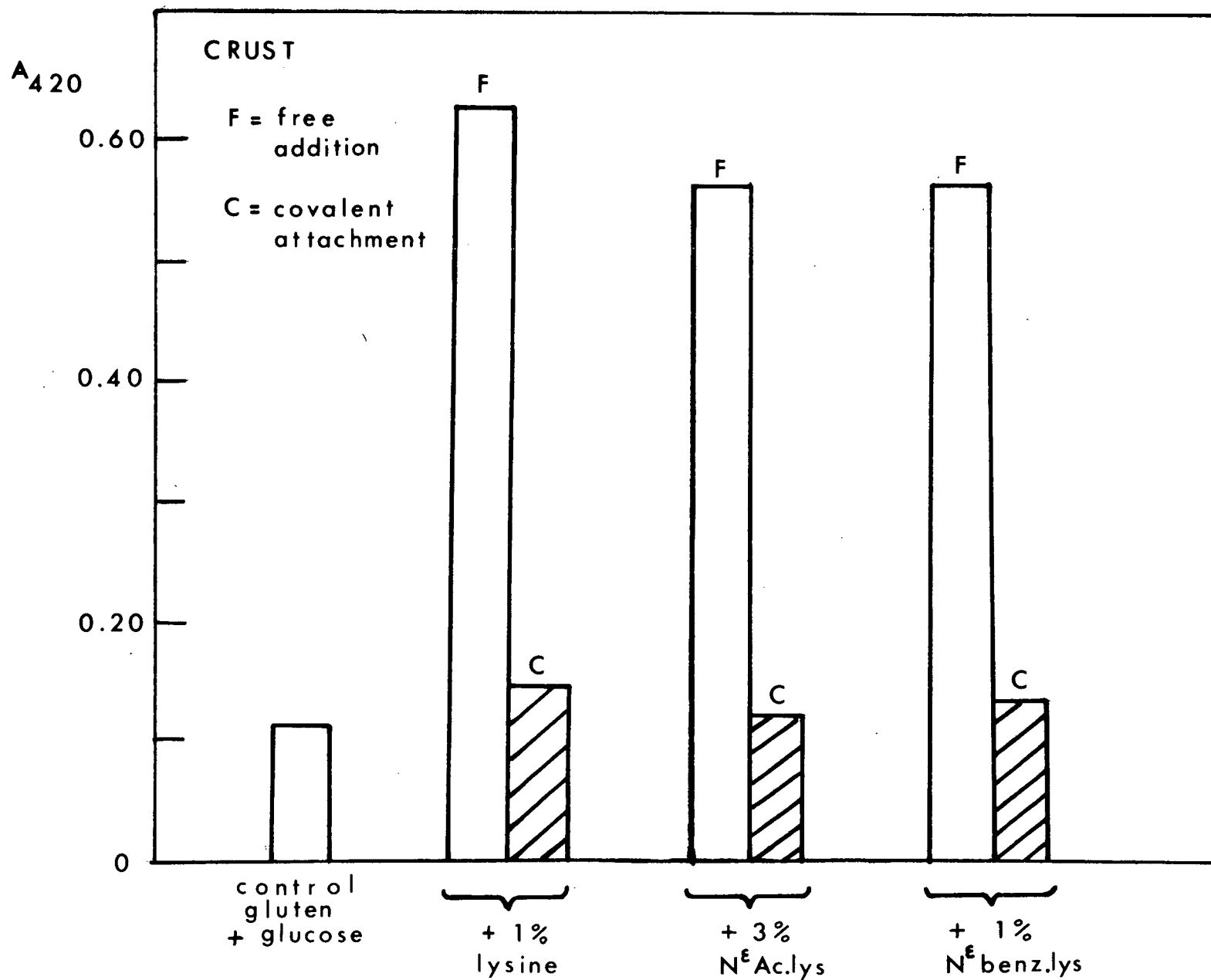
sample (crust only)	lysine (g/100 g)		% of added lysine remaining after baking
	before	after	
gluten	1.3	1.2	—
gluten + glucose	1.3	0.9	—
gluten + 1% lysine	2.5	1.3	8
gluten + 3% lysine	4.9	4.1	81
gluten + 1% lysine + glucose	2.5	0.7	0
gluten + 3% lysine + glucose	4.9	1.4	14
lysine-gluten (1%) + glucose	2.5	2.0	92
gluten + 3% N ^ε -acetyl lysine (AL)	4.0	3.1	70
gluten + 3% AL + glucose	4.0	1.7	30
AL-gluten (3%) + glucose	4.4	3.4	81
gluten + 1% N ^ε -benz.lysine (BL)	2.5	1.8	50
gluten + 3% BL	4.5	3.3	66
gluten + 1% BL + glucose	2.5	0.7	0
gluten + 3% BL + glucose	4.5	1.1	6
BL-gluten (1%) + glucose	2.6	2.3	108

lysine in the crust was destroyed. This suggests the predominant role of the Maillard reaction with reducing sugars in the destruction of lysine. When gluten samples were enriched by free addition of lysine, N^ϵ -acetyl lysine or N^ϵ -benzylidene lysine, very high percentages of the added lysine in the crust were lost after baking in the presence of glucose. In contrast, in samples which were enriched by covalent attachment of lysine or lysine derivative, very high percentages of the added lysine were recoverable. The percentages of added lysine remaining after baking in the presence of glucose were 92%, 81% and 108% for glutens covalently enriched with lysine, N^ϵ -acetyl lysine and N^ϵ -benzylidene lysine, respectively.

Figure 8 shows the absorbance of extracts obtained from the crust portion of different samples after baking in the presence of 10% glucose. The trend in color (A_{420}) of samples parallels the destruction of lysine. Samples which were enriched by covalent attachment showed only slightly higher absorbance readings than the control gluten, whereas samples which were enriched by free addition had much higher absorbance values. The latter samples were very dark brown in color when visualized with the eye, and were not acceptable aesthetically by the usual standards of bread crust color.

Lysine possesses an ϵ -amino group in addition to its α -amino group, and both groups can participate in the Maillard reaction with reducing sugars. Lysine causes the formation of more color in glucose-amino acid solutions than do monoaminomonocarboxylic acids such as alanine or monoaminodicarboxylic acids such as glutamic acid, and it can produce color over a wider pH range (Underwood *et al.*, 1959). The pronounced increase in color production by diamino acids cannot be

Figure 8. Absorbance of extracts of crust portions of free and covalently enriched glutens (baked with 10% glucose)



attributed only to the doubling of the number of the amino groups, nor to an algebraic sum of the color formed by the corresponding monoamino acids. Lysine produces six times more intense coloration than norleucine, an analogue with only an α -amino group. These observations may explain the much greater extent of browning and lysine destruction in the case of samples enriched by free addition, in which case both amino groups of lysine are available for reaction. In the covalently enriched sample, there is a net reduction of one amino group (α - or ϵ -amino of either the attached lysine or gluten) for every lysine residue attached by an amide bond. Thus, the available amino groups for the Maillard reaction are reduced in number in the covalently enriched sample. When N^ϵ -substituted derivatives such as N^ϵ -acetyl lysine or N^ϵ -benzylidene lysine are used, even less browning should occur since the lysine derivative has only one available amino group. The N^ϵ -substituent may, in addition, have either a stimulatory or an inhibitory effect on reactivity of the available α -amino group.

Finot et al. (1978) studied the reactivity of various N-substituted lysines toward browning and found that all the lysine derivatives were less reactive than free lysine. Dipeptides such as lysyl-alanine, alanyl-lysine and glycyl-lysine were about two times less reactive than lysine, while glutamyl-lysine was four times less reactive. α -Acyl derivatives were two times less reactive and ϵ -acyl derivatives were four times less reactive than lysine. ϵ -N-(α -glutamyl)-lysine was least reactive, being seven times less reactive than free lysine. These findings are in agreement with the ones reported in the present study. For example, as shown in Figure 8, gluten enriched by free addition of 3% N^ϵ -acetyl

lysine had lower absorbance (implying less browning) than gluten enriched by free addition of only 1% lysine.

The total lysine content of the crust portions is compared to the contents of DNBS-available and pepsin-pancreatin-digestible lysine in Table 33. Although the total lysine content in covalently enriched samples was higher than the content in samples enriched by free addition, this trend was not adhered to in the DNBS-available and pepsin-pancreatin-digestible lysine contents. Covalently enriched lysine and N^ε-acetyl lysine gluten samples had low values of both "availability" and "digestibility" by these in vitro test indices. As discussed in Part III-1 (in vitro evaluation), the added lysine residues in lysine gluten may be covalently attached through either peptide ($\alpha - \alpha$) or isopeptide ($\alpha - \epsilon$) bonds. In the latter type of linkage, the ϵ -amino group is not available for reaction with DNBS, nor is the isopeptide amide bond hydrolyzed by the proteases pepsin or pancreatin. Thus, available and digestible lysine contents are relatively low in comparison to total lysine content; yet these values are still higher than the corresponding values for the freely enriched sample, indicating that some of the lysine residues are attached by peptide bonds. In the case of N^ε-acetyl lysine gluten, the presence of the acetyl substituent blocks the ϵ -amino group from reacting with DNBS, thus giving low "available" content. The low "digestible" content was again probably due to the inability to quantitate the N^ε-acetyl lysine peak which overlapped with the proline peak in amino acid analysis.

Only covalently enriched N^ε-benzylidene lysine gluten yielded high values for total, available and digestible lysine contents. As

Table 33. Total, DNBS-available and pepsin-pancreatin-digestible lysine contents in crusts of enriched glutens after baking.

sample (crust only)	lysine (g/100 g total amino acids)		
	total	DNBS	PPD
unbaked gluten control	1.4	1.2	1.0
gluten	1.2	1.1	0.6
gluten + glucose	0.9	0.5	0.4
gluten + 1% lysine	1.3	1.0	1.0
gluten + 3% lysine	4.1	3.5	2.8
gluten + 1% lysine + glucose	0.7	0.5	0.3
gluten + 3% lysine + glucose	1.4	0.9	0.6
lysine-gluten (1%) + glucose	2.0	0.9	0.6
gluten + 3% N ^ε -acetyl lysine (AL)	3.1	1.5	1.0
gluten + 3% AL + glucose	1.7	< 0.2	0.1
AL-gluten (3%) + glucose	3.4	0.7	0.5
gluten + 1% N ^ε -benz.lysine(BL)	1.8	1.6	1.2
gluten + 3% BL	3.3	2.9	3.2
gluten + 1% BL + glucose	0.7	0.7	0.4
gluten + 3%BL + glucose	1.1	1.0	2.1
BL-gluten (1%) + glucose	2.3	1.4	1.1

mentioned earlier, the possible non-intentional removal of the benzylidene substituent under acidic conditions may have led to these results.

2. Comparison of whole (crust + crumb) samples

Table 34 shows the total lysine content of gluten and enriched gluten samples before and after baking in the presence of glucose, while Figure 9 depicts the absorbance of extracts obtained from these samples. Gluten samples covalently enriched with lysine, N^{ϵ} -acetyl lysine and N^{ϵ} -benzylidene lysine all had higher percentages of lysine remaining after baking when compared to the corresponding samples enriched by free addition. The smaller loss of lysine was accompanied by lighter color (lower absorbance) of extracts for covalently enriched samples. These trends for the whole (crust + crumb) gluten samples thus reflect findings observed from the crust fraction only of samples. Although, in general, much greater loss of lysine and darkening occurred in the crust and the differences between covalent and free enrichment were more marked when observing the isolated crust fraction, the results from the whole sample support the hypothesis that the covalently enriched lysine residues are more stable against destruction than lysine added in free form, during heating in the presence of a reducing sugar such as glucose.

The doughs prepared for the present study were mixtures of wheat gluten, glucose and water. The other ingredients usually included in a typical formulation for bread or other bakery products were not included to simplify interpretation of results. It is likely that components such as skim milk powder and wheat starch would affect the destruction

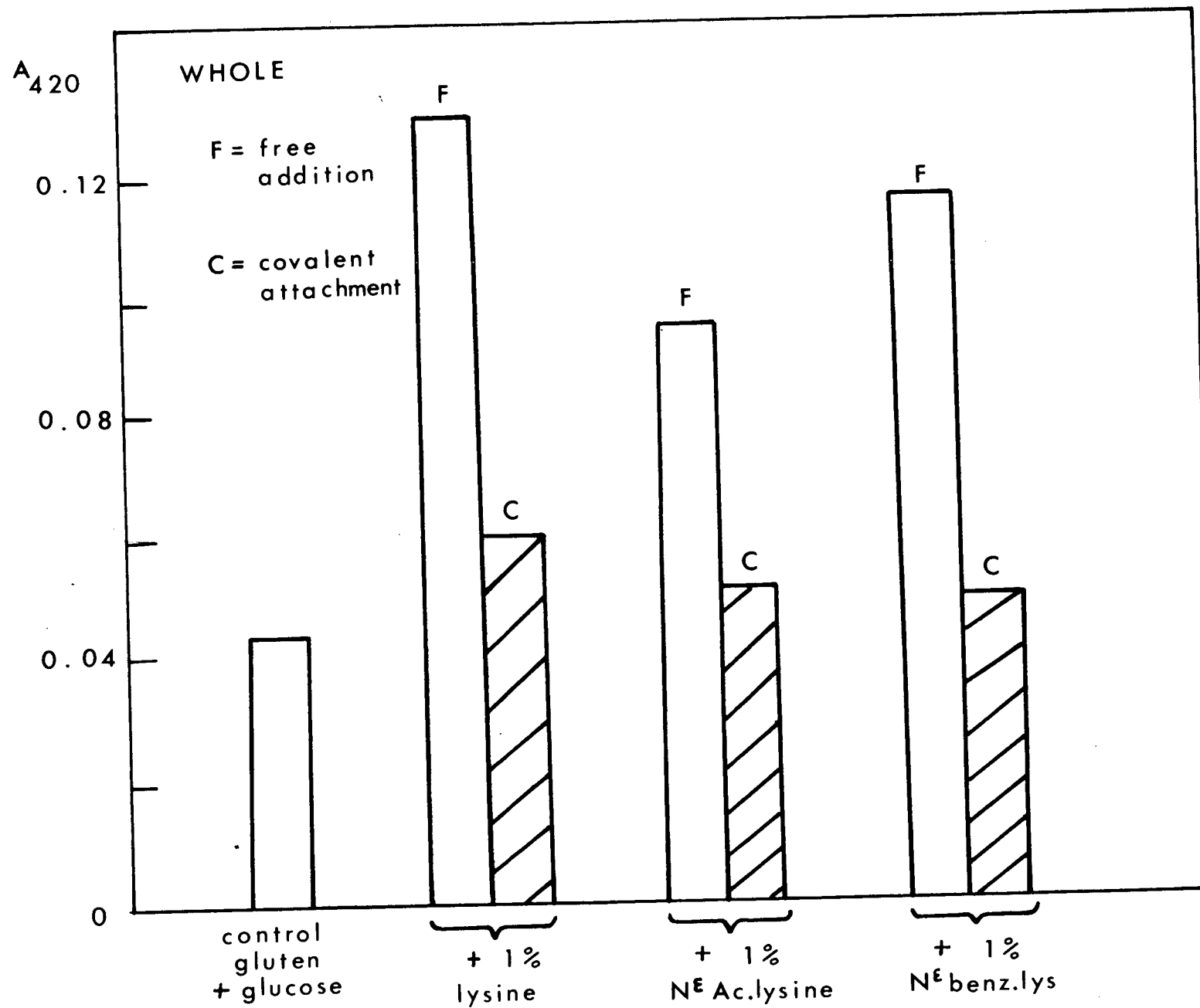
Table 34. Loss of lysine in enriched gluten and acid (0.05N HCl)-solubilized gluten samples after baking.

sample (whole)	lysine, g/100 g		% of added lysine remaining after baking
	before	after	
gluten	1.4	1.3	—
gluten + glucose	1.4	0.8	—
acid gluten + glucose	1.4	0.7	—
gluten + 1% lysine + glucose	2.5	1.3	45
lysine-gluten (1%) + glucose	2.5	1.6	73
gluten + 1% AL + glucose ^a	2.5	1.6	73
AL-gluten (1%) + glucose	2.5	2.0	110
gluten + 1% BL + glucose ^b	2.5	1.7	82
BL-gluten (1%) + glucose	2.5	2.0	110
acid gluten + 1% lysine + glucose	2.8	1.4	50
acid gluten + 4% lysine + glucose	5.6	2.2	36
lysine-acid gluten (1%) + glucose	2.8	2.0	93
lysine-acid gluten (4%) + glucose	5.6	2.8	50

^aAL = N^ε-acetyl lysine.

^bBL = N^ε-benzylidene lysine.

Figure 9. Absorbance of extracts of free and covalently enriched glutens (baked with 10% glucose)



of lysine during baking. Greater loss of supplemental lysine was observed in breads containing nonfat dry milk than breads lacking this ingredient (Jansen *et al.*, 1964a, 1964b). However, the conclusions obtained from the model studies presented here should be representative of the trends expected for more complex formulations of bakery products.

Lysine addition has been well-documented to cause significant darkening of crust color and occasionally changes in loaf volume (Ericson *et al.*, 1961; Jansen *et al.*, 1964a, 1964b; Ehle and Jansen, 1965). The loss of lysine (both protein-bound and added lysine) has been shown to be proportional to the baking time and to the percentage of crust which resulted (Jansen *et al.*, 1964a, 1964b). Nutritional value of bread after toasting decreases with increased extent of toasting (Tsen and Reddy, 1977), and high temperatures cause greater destruction of amino acids (Sabiston and Kennedy, 1957). When bread was fortified with 0.48% L-lysine (0.6% L-lysine.HCl) and 0.3% L-threonine, the baking losses of lysine and threonine were $5 \pm 6\%$ and $3 \pm 2\%$, respectively, in the crumb, $46 \pm 11\%$ and $54 \pm 8\%$, respectively in the crust, and $14 \pm 8\%$ and $15 \pm 5\%$, respectively, in the whole loaf (Murata *et al.*, 1979). The results reported in the present study confirm the greater percentage loss of lysine in the crust than in the whole loaf. Relatively large loss of lysine even in the whole loaf may be attributed to the small size of loaf (since doughs were prepared using 3 grams of gluten), which resulted in a large ratio of crust to crumb in the loaf.

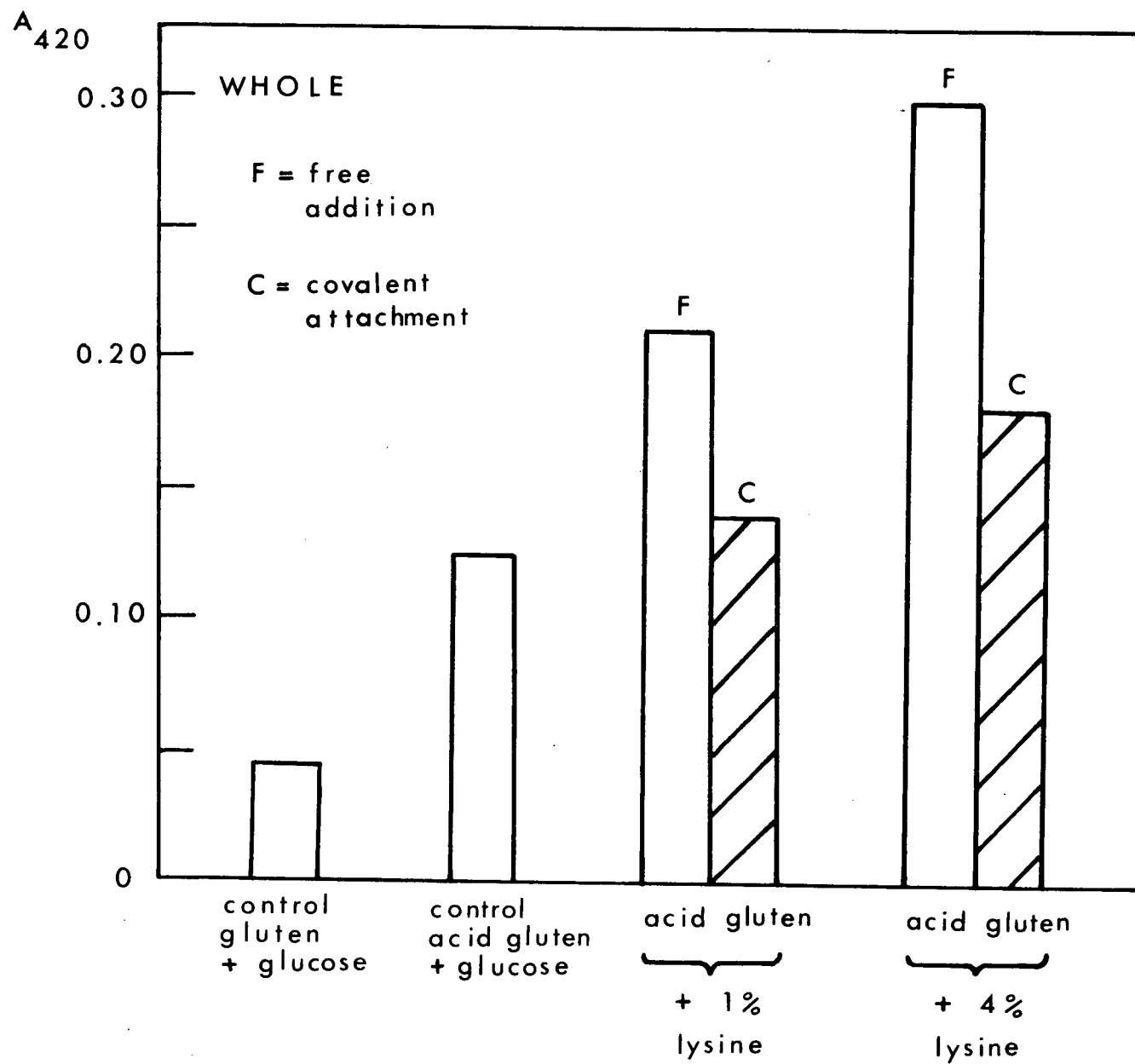
Table 34 also shows the loss of lysine in baked samples prepared from acid-solubilized gluten and acid-solubilized gluten enriched with 1% or 4% lysine. Covalently enriched lysine was more stable than freely

added lysine. When 1% lysine enrichment was used, the percentage of added lysine remaining after baking was higher in the acid-solubilized gluten samples than in corresponding gluten samples (50% versus 45% for freely added lysine, and 93% versus 73% for covalently added lysine). When lysine enrichment was undertaken at 4% level, only 36% and 50% of the freely added and covalently added lysine, respectively, remained after baking of the acid-solubilized gluten.

Comparison of absorbance of extracts (Figure 10) shows that the baked acid-solubilized gluten was darker than baked gluten control. Covalently enriched acid gluten samples were lighter in color than freely enriched acid gluten samples, both at the 1% and 4% levels of enrichment. However, both covalently and freely enriched acid gluten samples were darker than corresponding enriched gluten samples.

It is possible that the amide bond formed between lysine and acid-solubilized gluten, primarily an isopeptide bond ($\gamma - \alpha$ or $\gamma - \epsilon$) offered greater resistance of the attached lysine residues against Maillard browning reactivity than when the amide bond was of the peptide or isopeptide type found between lysine and gluten ($\alpha - \alpha$ or $\alpha - \epsilon$). It is known that reactivity toward Maillard browning destruction of amino acids depends on the side chain of the amino acid (Underwood et al., 1959), and different N-substituents of lysine have varying degrees of reactivity (Finot et al., 1978). The decreased loss of lysine in acid-solubilized gluten may have been a result of changed conformation of the gluten molecule by acid treatment, effects of the increased content of carboxyl groups on the proton transfer in reaction sequences such as the Amadori rearrangement or on the Strecker degradation of amino acids, or to the

Figure 10. Absorbance of extracts of free and covalently enriched acid-solubilized glutens (baked with 10% glucose).



protective effect of isopeptide linkages. Although lysine loss was reduced, the acid-solubilized gluten was darker in color, a result of perhaps changes in the other amino acids or of the gluten molecule as a whole. Even before baking, the acid-solubilized gluten was darker in color than gluten control.

Total, DNBS-available and pepsin-pancreatin-digestible lysine contents are compared in Table 35. Among gluten samples, highest contents were obtained for the covalently enriched N^ϵ -benzylidene lysine gluten, while covalently enriched N^ϵ -acetyl lysine gluten had high total lysine content but low available and digestible lysine contents. The covalently enriched lysine acid-solubilized gluten (1% and 4% levels of enrichment) had high values of total, available and digestible lysine contents. In general, these results are in agreement with those reported on the crust fractions of samples, and conclusions drawn regarding N^ϵ - blocking group lability and isopeptide bonds are similar.

In order to compare nutritional quality of the different samples after baking, samples were assayed using the Tetrahymena bioassay. The results are shown in Table 36. In the absence of glucose, the baked gluten, with a relative nutritive value of 57, had protein quality approximately equivalent to unbaked gluten. However, in the presence of added glucose, the relative nutritive value of gluten after baking decreased to 44. In a study comparing chemically measured lysine availability to Tetrahymena relative nutritive value, Warren and Labuza (1977) showed that the protozoan Tetrahymena is sensitive to the loss of protein quality due to nonenzymatic browning at advanced stages of browning, when the Tetrahymena test showed equivalent or greater losses of nutritional

Table 35. Total, DNBS-available and pepsin-pancreatin-digestible lysine contents in enriched gluten samples after baking.

sample (whole)	lysine, g/100 g total amino acids		
	total	DNBS	PPD
unbaked gluten control	1.4	1.2	1.0
unbaked acid gluten control	1.4	1.3	0.6
gluten	1.3	1.2	0.9
gluten + glucose	0.8	0.6	0.6
acid gluten + glucose	0.7	0.6	0.5
gluten + 1% lysine + glucose	1.3	0.7	0.6
lysine-gluten (1%) + glucose	1.6	0.8	0.8
gluten + 1% AL + glucose ^a	1.6	< 0.2	0.5
AL-gluten (1%) + glucose	2.0	0.7	0.7
gluten + 1% BL + glucose ^b	1.7	1.0	0.8
BL-gluten (1%) + glucose	2.0	1.4	1.3
acid gluten + 1% lysine + glucose	1.4	0.7	0.6
acid gluten + 4% lysine + glucose	2.2	0.8	0.8
lysine-acid gluten (1%) + glucose	2.0	1.5	0.9
lysine-acid gluten (4%) + glucose	2.8	1.9	1.5

^aAL = N^ε-acetyl lysine.

^bBL = N^ε-benzylidene lysine.

Table 36. Relative nutritive values (RNV) of enriched gluten samples after baking.

sample (whole)	relative nutritive value ^a
gluten	57
gluten + glucose	44
acid gluten + glucose	45
gluten + 1% lysine + glucose	50
lysine-gluten (1%) + glucose	63
gluten + 1% N ^ε -acetyl lysine + glucose	53
N ^ε -acetyl lysine-gluten (1%) + glucose	75
gluten + 1% N ^ε -benz.lysine + glucose	58
N ^ε -benz.lysine-gluten (1%) + glucose	88
acid gluten + 1% lysine + glucose	52
acid gluten + 4% lysine + glucose	81
lysine-acid gluten (1%) + glucose	61
lysine-acid gluten (4%) + glucose	91

^a relative nutritive value = 100 x (organism count for sample/ organism count for casein)

value compared to the fluorodinitrobenzene chemical assay. At early stages of browning, however, less loss was detected by Tetrahymena than by the chemical test, suggesting possibly that some of the early Maillard reaction products may be biologically available to Tetrahymena, or that some loss of lysine and other amino acids may not be detrimental to its growth.

Enrichment of gluten samples by free addition of lysine, N^ϵ -acetyl lysine, or N^ϵ -benzylidene lysine before baking resulted in some improvements in protein quality, the relative nutritive values being increased to 50 to 58. Covalent enrichment with lysine or lysine derivatives improved relative nutritive values more effectively, with the best growth occurring in the test culture medium containing covalently enriched N^ϵ -benzylidene lysine gluten. Relative nutritive value in this case was 88, in comparison to a value of 100 for an unbaked casein reference protein. Covalent enrichment of acid-solubilized gluten also significantly improved protein quality. At a 4% level of enrichment before baking, the baked sample had relative nutritive value of 91.

The improvement of relative nutritive values by addition of lysine derivatives as well as of lysine, and by enrichment of acid-solubilized gluten as well as of gluten, demonstrates the biological availability of these N^ϵ -substituents of lysine and of lysine attached by isopeptide bonds, at least in terms of availability as sources of lysine for growth of the protozoan Tetrahymena. The biological availability of these products in conjunction with their reduced sensitivity toward degradative reactions such as Maillard browning demonstrates great potential advantages including covalently attached N^ϵ -substituted lysine derivatives for

stable enrichment of food proteins.

CONCLUSIONS

The results of this study demonstrate the feasibility of improving the protein nutritional quality of wheat gluten through covalent attachment of limiting amino acids by a chemical reaction aided by the coupling reagent, EDC. The efficiency of attachment was found to depend on several conditions, particularly pH of the reacting medium, and concentration and type of the reactants. The recovery of enriched product depended primarily on the effects of solubilization of gluten prior to the carbodiimide reaction. Threonine, lysine and N^ϵ -protected derivatives such as N^ϵ -benzylidene lysine and N^ϵ -acetyl lysine could be efficiently attached to pepsin-hydrolyzed gluten, but product yields were low. The efficiency of attachment was decreased using gluten as the reactant, but product yields in this case were high. Solubilization of gluten by mild acid hydrolysis resulted in efficient attachment (up to 20-fold increase in lysine content) with high recovery of product (approximately 90% yield).

In vitro availability and digestibility tests suggested the formation of isopeptide bonds as well as peptide bonds for the covalent attachment of the amino acids. Isopeptide bonds were indicated involving the γ -carboxyl groups of acid-solubilized gluten and the ϵ -amino group of lysine. Peptide (α - α) bonds predominated when N^ϵ -protected derivatives of lysine were coupled to gluten or pepsin-hydrolyzed gluten. The Schiff's base linkage binding the benzylidene group to the ϵ -amino group was suggested to be much more labile than the amide linkage binding the acetyl group to the ϵ -amino group.

Although the isopeptide bonds and N^ϵ -blocked amino groups were not available by the in vitro tests, microbial evaluation using the protozoan Tetrahymena demonstrated the biological availability of gluten covalently enriched with lysine, N^ϵ -acetyl lysine as well as N^ϵ -benzylidene lysine. The bioassay could not detect any difference, with respect to the protein quality for Tetrahymena growth, between covalently or freely enriched glutens and reference casein. The enriched glutens were all nutritionally superior to gluten. Further, more thorough biological evaluation using test animals and eventually humans is required to confirm these preliminary results and to assess the possible effects of covalent versus free enrichment.

Greater stability of covalently bound lysine compared to free lysine was indicated in model baking studies of the enriched products. These results illustrate the advantage of covalent attachment for enrichment of food proteins susceptible to degradative reactions such as Maillard browning.

Although some conclusions can be made from this study regarding the influence of reaction parameters on the attachment, further studies are needed to optimize the conditions for economical feasibility in large scale applications. The reaction conditions can be rigidly controlled in theory to obtain a protein with the ideal amino acid composition. However, it is much more practical to manipulate the conditions of the reaction for maximal attachment of limiting amino acids, and subsequently mix the enriched proteins with the original poor quality protein to create the "ideal" protein.

The choice of solubilization treatment of protein or the need for protecting groups to block functional side chains depends on the purpose of utilization of the enriched protein. The acetyl group has been recommended by some workers as an ideal protecting substituent due to its natural existence in biological systems. Yet the benzylidene group may have the advantage of being easily removed when no longer required. The use of ϵ -amino protected lysine may be recommended for fortification of foods susceptible to lysine destruction via amino group reactivity. Otherwise, the use of lysine is obviously more economical and convenient. Although acid solubilization results in effective attachment with high product yields, the convenience of using gluten without prior treatment may override, under some circumstances, the disadvantage of lower extent of attachment. Pepsin hydrolysis also results in effective attachment of amino acids. The low product yields may possibly be overcome if the dialysis technique used in the present study is replaced by methods such as precipitation, ultra-filtration or even dialysis with tubing offering greater retention of intermediate molecular weight compounds. Different functional and physiological properties may be expected depending on the choice and prior treatment of reactants.

The term "PROLYSINE" was suggested by Finot et al. (1978) to describe biologically available derivatives of lysine. Prolysines were recommended to be advantageous as additives in foods rich in reducing sugars and for utilization in parenteral nutrition. The results from the study presented here also demonstrate the advantages of prolysines such as N^{ϵ} -acetyl lysine and N^{ϵ} -benzylidene lysine for nutritional improvement

of proteins. Further potential improvements are gained by covalent attachment of these prolysines or other limiting amino acids to food proteins. The formation of an isopeptide bond for the attachment of lysine in effect confers on the attached lysine the same advantages of the N^ε-substituted prolysines. In addition, covalent attachment may offer further improvements such as protection against losses during processing steps including washing or discarding of cooking juices, or better utilization by the digestive system. Improvement in existing properties or development of new functional properties may be supplementary advantages achieved through controlled attachment of specific amino acids. The confirmation of the great potential uses of covalent attachment of limiting nutrients to food systems awaits further research in this relatively under-explored novel field of food science.

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APPENDIX

I. Fractional Factorial Experimental Designs (Taguchi, 1957)

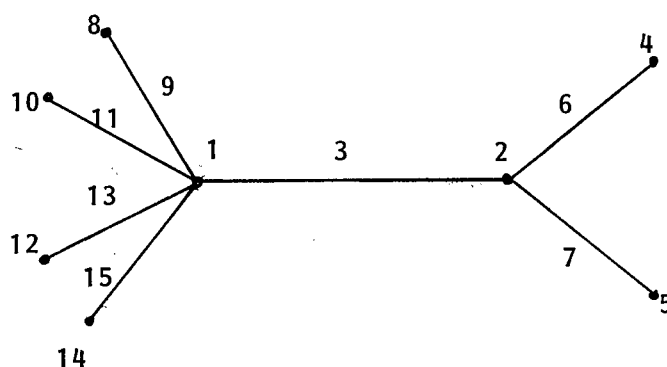
1. Two-level fractional factorial experiments ($L_{16}(2^{15})$) for selecting influential factors

expt. no.	factor level assigned for column														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
2	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2
3	1	1	1	2	2	2	2	1	1	1	1	2	2	2	2
4	1	1	1	2	2	2	2	2	2	2	2	1	1	1	1
5	1	2	2	1	1	2	2	1	1	2	2	1	1	2	2
6	1	2	2	1	1	2	2	2	2	1	1	2	2	1	1
7	1	2	2	2	2	1	1	1	1	2	2	2	2	1	1
8	1	2	2	2	2	1	1	2	2	1	1	1	1	2	2
9	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
10	2	1	2	1	2	1	2	2	1	2	1	2	1	2	1
11	2	1	2	2	1	2	1	1	2	1	2	2	1	2	1
12	2	1	2	2	1	2	1	2	1	2	1	1	2	1	2
13	2	2	1	1	2	2	1	1	2	2	1	1	2	2	1
14	2	2	1	1	2	2	1	2	1	1	2	2	1	1	2
15	2	2	1	2	1	1	2	1	2	2	1	2	1	1	2
16	2	2	1	2	1	1	2	2	1	1	2	1	2	2	1

Assignment of factors to columns and possible interaction detection:

- (a) Attachment of N^{ϵ} -benzylidene lysine to pepsin-hydrolyzed gluten.

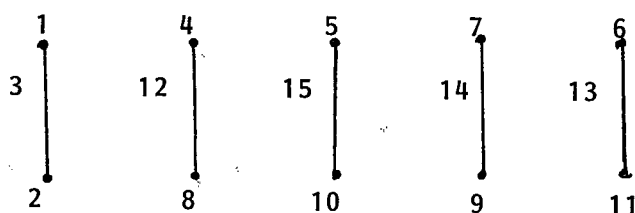
Scheme to detect interactions



<u>Factor or Interaction</u>	<u>column</u>
gluten concentration	1
reaction time	2
reaction temperature	4
pH	8
pepsin hydrolysis time	10
EDC amount	12
N ^ε -benzylidene lysine amount	14
reaction time x gluten concn	3

(b) Attachment of N^ε-acetyl lysine to gluten

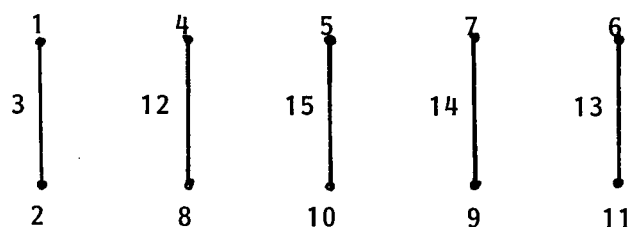
Scheme to detect interactions:



<u>Factor or Interaction</u>	<u>column</u>
gluten concentration	1
sodium stearate amount	2
EDC amount	4
pH	5
reaction time	7
activation time	8
reaction temperature	9
N ^ε -acetyl lysine amount	10
gluten x stearate	3
EDC x activation time	12
pH x N ^ε -acetyl lysine	15
reaction time x temperature	14

(c) Attachment of threonine to pepsin-hydrolyzed gluten

Scheme to detect interactions:

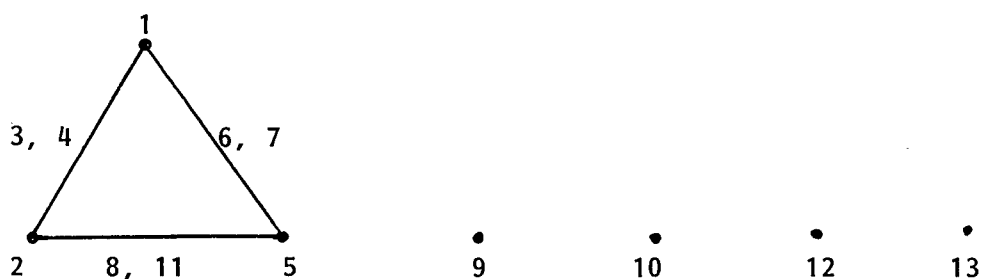


<u>Factor of Interaction</u>	<u>Column</u>
pepsin hydrolysis time	1
EDC amount	2
gluten concentration	4
pH	5
ionic strength	6
reaction time	7
theonine amount	8
reaction temperature	9
activation time	10
polarity	11
gluten concn x threonine	12
reaction time x temperature	14
pepsin time x EDC	3
ionic strength x polarity	13

2. Three-level fractional factorial experiments ($L_{27}(3^{13})$) for selecting influential factors and optimization

expt.	factor level assigned for column												
no.	1	2	3	4	5	6	7	8	9	10	11	12	13
1	1	1	1	1	1	1	1	1	1	1	1	1	1
2	1	1	1	1	2	2	2	2	2	2	2	2	2
3	1	1	1	1	3	3	3	3	3	3	3	3	3
4	1	2	2	2	1	1	1	2	2	2	3	3	3
5	1	2	2	2	2	2	2	3	3	3	1	1	1
6	1	2	2	2	3	3	3	1	1	1	2	2	2
7	1	3	3	3	1	1	1	3	3	3	2	2	2
8	1	3	3	3	2	2	2	1	1	1	3	3	3
9	1	3	3	3	3	3	3	2	2	2	1	1	1
10	2	1	2	3	1	2	3	1	2	3	1	2	3
11	2	1	2	3	2	3	1	2	3	1	2	3	1
12	2	1	2	3	3	1	2	3	1	2	3	1	2
13	2	2	3	1	1	2	3	2	3	1	3	1	2
14	2	2	3	1	2	3	1	3	1	2	1	2	3
15	2	2	3	1	3	1	2	1	2	3	2	3	1
16	2	3	1	2	1	2	3	3	1	2	2	3	1
17	2	3	1	2	2	3	1	1	2	3	3	1	2
18	2	3	1	2	3	1	2	2	3	1	1	2	3
19	3	1	3	2	1	3	2	1	3	2	1	3	2
20	3	1	3	2	2	1	3	2	1	2	2	1	3
21	3	1	3	2	3	2	1	3	2	1	3	2	1
22	3	2	1	3	1	3	2	2	1	3	3	2	1
23	3	2	1	3	2	1	3	3	2	1	1	3	2
24	3	2	1	3	3	2	1	1	3	2	2	1	3
25	3	3	2	1	1	3	2	3	2	1	2	1	3
26	3	3	2	1	2	1	3	1	3	2	3	2	1
27	3	3	2	1	3	2	1	2	1	3	1	3	2

Scheme to detect interactions:



Assignment of factors to columns and possible interaction detection for attachment of threonine to gluten:

<u>Factor or Interaction</u>	<u>column</u>
EDC amount	1
threonine amount	2
gluten concentration	5
pH	9
reaction time	10
reaction temperature	12
polarity	13
EDC x threonine	3, 4
EDC x gluten	6, 7
threonine x gluten	8, 11

3. Four-level fractional factorial experiments ($L_{16}(4^5)$) for optimization or confirmation of optimum.

expt.	factor level assigned for column				
no.	1	2	3	4	5
1	1	1	1	1	1
2	1	2	2	2	2
3	1	3	3	3	3
4	1	4	4	4	4
5	1	2	3	4	5
6	2	2	1	4	3
7	2	3	4	1	2
8	2	4	3	2	1
9	3	1	3	4	2
10	3	2	4	3	1
11	3	3	1	2	4
12	3	4	2	1	3
13	4	1	4	2	3
14	4	2	3	1	4
15	4	3	2	4	1
16	4	4	1	3	2

Assignment of factors to columns:

- (a) Attachment of N^ϵ -acetyl lysine to gluten

<u>Factor</u>	<u>Column</u>
pH	1
N^ϵ -acetyl lysine amount	2
reaction time	3

- (b) Attachment of threonine to pepsin-hydrolyzed gluten

<u>Factor</u>	<u>Column</u>
EDC amount	1
threonine amount	2
gluten concentration	3

II. Simplex Method for Optimization (Morgan and Deming, 1974)

1. Calculate the initial simplex containing (k + 1) vertices

The initial simplex D_0 is a matrix with (k) columns and (k + 1) rows, where 0_k = the number of factors or variable.

eg. for k = 3,

$$D_0 = \begin{vmatrix} 0 & 0 & 0 \\ p & q & q \\ q & p & q \\ q & q & p \end{vmatrix}$$

where 0 = lower boundary value

$$p = 1/k\sqrt{2} [(k-1) + (\sqrt{k+1})]$$

$$q = 1/k\sqrt{2} [(\sqrt{k+1}) - 1]$$

2. Based on initial simplex results, calculate reflection R and measure its response.

$$R = \bar{P} + (\bar{P} - W) \text{ where } W = \text{worst response}$$

\bar{P} = centroid after excluding W

3. Calculation of next simplex based on response at R, Y_R .
(see following scheme)

$$\bar{\bar{P}} + C(\bar{P} - W) \text{ where } C = 1.0 \text{ for } R$$

$$0.5 \quad C_r$$

$$0.25 \quad C_{r'}$$

$$2.0 \quad E$$

$$-0.5 \quad C_w$$

$$-0.25 \quad C_{w'}$$

Calculation of next simplex vertex to replace W for maximization of response:

