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Preparation and Nutritional Properties of Caseins Covalently Modified with Sugars. Reductive Alkylation of Lysines with Glucose, Fructose, or Lactose

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Reducing sugars were attached to the ϵ-amino lysyl residues of casein in the presence of sodium cyanoborohydride, forming stable amine linkages. At pH 8.0 and 37 °C for 120 h in the presence of 1000 M excess of sugar, the degree of modification was 80% with glucose, 62% with fructose, and 17% with lactose. Compared to native casein, these reductively formed sugar derivatives of casein were shown to have lower in vitro digestibility by α-chymotrypsin and lower nutritive values in rat feeding experiments. Severe growth depression was observed in rats fed with the glucose-derivatized casein. Addition of lysine to the diet containing glucose-derivatized casein (71% modified) partially alleviated the growth-depressing effects. Moderate growth depression was observed in rats fed with the fructose-derivatized casein or with the lactose-derivatized casein. Since these three derivatives were not equally modified, the observed rat growth responses may be due to the decreased availability of modified lysine as well as to the nature of the attached sugar.

In developing new low-cost, high-quality protein foods such as beverages and bars (Altschul, 1969), proteins need modifying in order to obtain products with desirable characteristics (Feeney, 1977). Properties which can be affected by chemical modification include solubility at different pH values, susceptibility to heat denaturation, stability during storage, and extent of hydration and gel formation.

Evans et al. (1971) found that the polymer content of acyl β-casein derivatives increased with increasing n-alkyl chain length, suggesting aggregation via hydrophobic bonding. The n-hexanoyl, n-octanoyl, and n-decanoyl derivatives associated more strongly with increasing concentration and remained aggregated after dilution or equilibration at low temperature. Acetyl and propionyl β-caseins formed monomer–polymer systems similar to native β-casein, but they associated less strongly than β-casein due to their enhanced negative charge. Studies of the functional properties of acetylated and succinylated proteins in model systems have revealed improved heat stability, dispersion, foaming, and emulsification capacity (Groninger, 1973). Lee et al. (1978) have shown that methylation of as many as half of the side chain amino groups of lysines in casein does not lower the availability of lysine when the modified casein is fed to rats. Demethylation of methylated lysines was seen in the blood.

Attachment of hydrophilic groups (e.g., carbohydrates, phosphates) to proteins should also change their solubility, viscosity, hydration, and gel-forming characteristics. Although chemical phosphorylation is used mainly in the synthesis of nucleotides (Pettit, 1972), phosphorylation of proteins by chemical means has not been investigated extensively due to the harsh reaction conditions commonly used.

A number of methods for the covalent attachment of carbohydrates to proteins have been developed (Gray, 1974; Marshall and Rabinowitz, 1975; Krantz et al., 1976). The products obtained were used in immunological as well as enzyme heat stability studies. Marshall and Rabinowitz (1975) prepared soluble enzyme–carbohydrate conjugates by coupling trypsin, α-amylase, and β-amylase to cyanogen bromide-activated dextran. The conjugates were more stable to heat than the respective native enzymes. Loss of trypsin activity by autolytic hydrolysis was also decreased by attachment of carbohydrate. Attachment of D-galactosides to proteins enhanced rabbit liver membrane binding by several orders of magnitude, while attachment of D-glucosides enhanced binding to a variable extent depending on the method of linkage. Coupling of thioglycosides to proteins has been achieved by amidination, diazo coupling, and amide formation (Krantz et al., 1976).

Gray (1974) utilized a modification of the reductive alkylation reaction described by Means and Feeney (1968) for the direct coupling of reducing oligosaccharides to proteins with sodium cyanoborohydride in aqueous solution at pH 7. This procedure relies on the ability of the cyanoborohydride anion to selectively reduce the Schiff base formed between the carbonyl group of reducing sugars and the free amino groups of proteins. The secondary amine linkage thus formed is stable to acid hydrolysis. This reduction by sodium cyanoborohydride was shown

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by Borch et al. (1971) to proceed most readily at pH 6–8 where the reduction of aldehydes and ketones is negligible. When the Schiff base was not reduced, subsequent nonenzymatic browning reactions occurred, including rearrangements, eventual scissions and multiple products (Carpenter, 1973; Feeney et al., 1975).

In the present study, casein ϵ-amino lysyl residues were derivatized with several reducing sugars via the reductive alkylation reaction in the presence of sodium cyanoborohydride. This procedure was chosen because the reaction is relatively simple, specific, and well-characterized. The nutritive value of these sugar-derivatized caseins was then evaluated by determining their susceptibility to hydrolysis by α-chymotrypsin and in growth trials with young rats.

MATERIALS AND METHODS

Materials. Vitamin-free casein was purchased from Nutritional Biochemicals Corp., sodium cyanoborohydride from Aldrich Chemical Co., α-D-glucose (cerelose) from Corn Products Co., β-D-fructose, α-lactose hydrate, and L-lysine hydrochloride from Sigma Chemical Co. All other reagents were of analytical grade.

Effect of pH on Reductive Alkylation with Glucose. Casein (0.5 g) was dissolved in 25 mL of one of three buffers (pH 7.0 and 8.0, 0.2 M potassium phosphate; pH 9.0, 0.1 M Na_2CO_3–$NaHCO_3$) containing 5% dioxane with warming to about 40 °C. Dioxane was included at the 5% level as a dispersion medium for the casein; higher levels, e.g., 20%, have also been used for the reagents in other studies (Fretheim et al., 1979). The casein solutions were mixed with 2 g of glucose and 0.3 g of $NaCNBH_3$ and kept at 37 °C in a thermostatically controlled oven. Five-milliliter aliquots were taken at various time intervals, dialyzed against H_2O, and lyophilized.

Preparation of Glucose-Derivatized Casein. Casein (300 g) was dissolved with warming to about 40 °C in 3 L of 0.2 M potassium phosphate buffer, pH 8.0, containing 5% dioxane. The casein solution was then mixed with 150 g of glucose and 40 g of $NaCNBH_3$ and kept at 37 °C for 120 h. NaCl was added to give a final concentration of 0.1 M. The solution was dialyzed against three changes of 0.1 M NaCl solution and against several changes of distilled H_2O. The glucose-derivatized casein [containing ϵ-*N*-1-(1-deoxyglucitol)lysine] was precipitated by addition of HCl to pH 4.5, freeze-dried, and ground to a fine powder. Another 300 g of casein was similarly treated without the addition of $NaCNBH_3$.

For a larger preparation of glucose-derivatized casein, 800 g of casein in 10 L of buffer was reacted with 400 g of glucose and 100 g of $NaCNBH_3$ at 37 °C for 120 h and further processed as above.

Preparation of Fructose-Derivatized Casein. The same procedure was followed as in the preparation of glucose-derivatized casein except that 150 g of fructose was used for 300 g of casein.

Preparation of Lactose-Derivatized Casein. The same procedure was followed as in the preparation of glucose-derivatized casein except that 300 g of lactose was used for 300 g of casein.

Analytical Procedures. Free amino groups in casein samples were determined by the 2,4,6-trinitrobenzenesulfonic acid (TNBS) method as described by Fields (1972). For amino acid analyses, casein samples were hydrolyzed in 6 N HCl in vacuo at 110 °C for 22 h. The hydrolyzed samples were analyzed on a Technicon Autoanalyzer using the pH 5 buffer system (Williams and Woodhouse, 1967). Protein concentrations were determined by the biuret method (Clark, 1964).

Table I. Percentage Composition of Diets

casein[a] or modified casein	10
L-Lys·HCl[b,c]	0
glucose[d]	74
corn oil[e]	8
mineral mix[f]	6
vitamin mix[g]	2

[a] Vitamin-free, Nutritional Biochemicals Corp., Cleveland, OH. [b] Sigma Chemical Co., St. Louis, MO. [c] Incorporated at 0.8% at the expense of glucose in one diet. [d] Cerelose, Corn Products Co., International, Englewood Cliffs, NJ. [e] Mazola, Corn Products Co., New York. [f] Supplied the following (g/kg diet): $CaCO_3$, 18.0; K_2HPO_4, 19.5; $CaHPO_4$, 3.6; NaCl, 10.08; $FeSO_4 \cdot 7H_2O$, 1.5; $MgSO_4$, 3.88; KI, 0.015; $ZnCO_3$, 0.048; $CuSO_4 \cdot 5H_2O$, 0.018; $MnSO_4 \cdot H_2O$, 0.14. [g] Supplied the following (mg/kg diet): inositol, 500; ascorbic acid, 100; calcium pantothenate, 50; thiamin hydrochloride, 30; pyridoxine hydrochloride, 30; nicotinic acid, 30; menadione, 25; riboflavin, 10; *p*-aminobenzoic acid, 10; folic acid, 0.6; biotin, 0.25; cyanocobalamin, 0.03; choline chloride, 14.3; α-tocopherol, 54 000 IU; vitamin A palmitate, 15 000 IU; cholecalciferol, 1500 IU.

Diets and Animals. The percentage composition of diets is listed in Table I. Weanling Sprague-Dawley male rats, housed individually in suspended cages, were fed a stock diet (Ralston Purina Co.) for 2 days and then divided into groups of five rats each with approximately equal mean initial weights (about 70 g in experiment 1 and 64 g in experiment 2). Body weight and ad libitum food intake were recorded two–three times weekly throughout the test periods.

In Vitro Enzyme Digestion. The initial rates of hydrolysis of unmodified and sugar-derivatized caseins by α-chymotrypsin were determined by measuring with time the amount of amino groups liberated with the TNBS method (Fields, 1972). Hydrolysis was done with 7.5 nM α-chymotrypsin and 0.1% casein in 0.02 M sodium borate buffer, pH 8.2 at 38 °C.

The extents of hydrolysis were determined in the same manner as above except the enzyme concentration was 22.5 nM during the first 24 h and 45 nM during the second 24 h. This high enzyme concentration insures hydrolysis of all susceptible bonds. After 48 h, aliquots were taken to measure the liberated amino groups by the TNBS method (Fields, 1972).

Statistical Analysis. Growth and food intake data were subjected to analysis of variance, and treatment means were compared using Duncan's Multiple Range Test (Hicks, 1964) at the 5% level of probability.

RESULTS

Preparation and Analysis of Sugar Casein Derivatives. The effect of pH on the rate of coupling of glucose is shown in Table II. The rates were essentially the same at pH 8.0 and 9.0. Under these conditions, about 80% of the available lysyl residues were derivatized after 110 h at 37 °C. At pH 7.0, the rate of substitution was slightly decreased at all time intervals examined. The present study was therefore done at pH 8.0 since the reduction of carbonyl groups of reducing sugars by sodium cyanoborohydride is negligible at this pH (Borch et al., 1971).

During the preparations of glucose, fructose, and lactose casein derivatives, samples were taken at various time periods and analyzed for losses in lysine. The rate of coupling was quite dependent on the nature of the reducing sugars as shown in Figure 1. The monosaccharides glucose and fructose were coupled at a much faster rate than the disaccharide lactose under the same conditions. The glucose–casein reaction mixture became extremely

Table II. Effect of pH on the Rate of Coupling of Glucose to ϵ-Amino Groups of Lysyl Residues of Casein in the Presence of Cyanoborohydride[a]

time, h	% loss in lysines at pH 7.0[b]	pH 8.0[b]	pH 9.0[c]
control[d]	0	0	0
12	18	24	17
35	34	45	44
110	69	80	82

[a] Reaction done at 37 °C. Losses in lysines followed by TNBS assay (Fields, 1972). Each value represents the average of two determinations. [b] Potassium phosphate buffers, 0.2 M. [c] Sodium carbonate buffer, 0.1 M. [d] Controls represent zero time samples, i.e., immediately before addition of $NaCNBH_4$. A separate control was run at each pH value.

Table III. In Vitro Digestibility of Sugar-Casein Derivatives at pH 8.2 and 38 °C

substrate	degree of modificat., %	rel rates[a]	rel extent[b]
unmodified		1.0	1.0
modified casein modifying groups			
glucose	30	0.47	0.91
	53	0.24	0.69
	67	0.25	0.64
	80	0.25	0.60
fructose	20	0.41	0.87
	37	0.30	0.74
	52	0.30	0.66
	69	0.26	0.60

[a] α-Chymotrypsin, 7.5 nM. [b] α-Chymotrypsin (45 nM) for 48 h.

viscous at 120 h, indicating some changes in hydration properties of the 80% modified casein.

In Vitro Enzyme Digestion. Table III shows the relative initial rates and extents of α-chymotrypsin catalyzed digestion of sugar casein derivatives. α-Chymotrypsin was used rather than trypsin because alkylation of lysyl residues greatly decreases the rate of hydrolysis of the lysyl peptide bond by trypsin but has no direct effect on the hydrolysis of peptide bonds by α-chymotrypsin (Lin et al., 1969; Galembeck et al., 1977). Any changes in digestibility with α-chymotrypsin would therefore be affected by causes other than changes in susceptibility of a particular type of peptide bond. At pH 8.2 and 38 °C there was a decrease in both initial rates and extents of digestion of derivatized caseins as compared to native casein. As the degree of modification increased, the initial rates and extent of digestion decreased. The nature of the reducing sugar did not seem to greatly affect the initial rates and extents of digestion. There was no significant difference in initial rates or in the extents of digestion

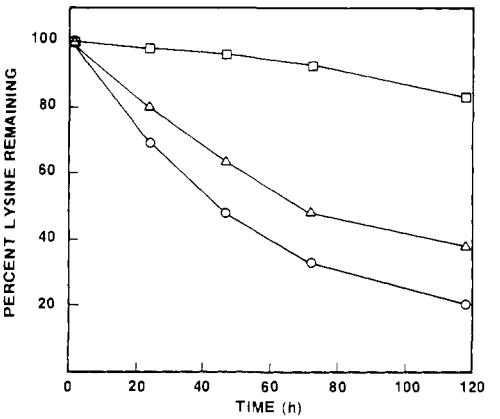

Figure 1. The rate of coupling glucose (○), fructose (△), and lactose (□) to the ϵ-amino group of lysyl residues of casein in the presence of cyanoborohydride at 37 °C in 0.2 M potassium phosphate buffer, pH 9.0. Losses in lysine were determined by amino acid analysis.

between 69% fructose-derivatized casein and 67% glucose-derivatized casein nor between 52% fructose-derivatized casein and 53% glucose-derivatized casein.

Growth. The growth response of rats receiving diets supplying 10% native or modified caseins in experiment 1 is summarized in Table IV. All three casein preparations treated with glucose, fructose, or lactose in the absence of sodium cyanoborohydride supported normal rat growth. Significantly lower growth rates were observed in rats fed the diets containing sugar casein derivatives prepared by treatment with glucose, fructose, or lactose in the presence of sodium cyanoborohydride (Table IV). The glucose-derivatized casein (80% modified) group gained only 3.5 g/rat in 2 weeks, while the fructose-derivatized casein (62% modified) and lactose-derivatized casein (17% modified) groups gained an average of about 22 g/rat, as compared to the 34 g/rat for the control. Food intake and food efficiency were also lowered for the sugar casein derivative groups. All three sugar casein derivative groups exhibited some diarrhea, the lactose-derivatized casein group being the most severe. The condition of diarrhea could have been due to any one of several causes, e.g., some fermentation of modified products in the intestines or osmotic effects resulting from incomplete absorption of partially degraded products.

In order to determine whether reduced availability of lysine was responsible for the reduced growth rate, a second feeding trial was conducted with or without lysine addition (0.8% L-lysine hydrochloride) to a diet containing 10% glucose-derivatized casein (71% modified). Results of experiment 2 are presented in Table V. The amount of glucose coupled in experiment 2 was not as high as that used in experiment 1. Consequently a higher weight gain of 14.8 g/rat was observed for the unsupplemented glu-

Table IV. Growth Response of Rats Fed Diets Supplying 10% Casein Treated with Glucose, Fructose, or Lactose in the Absence or Presence of Sodium Cyanoborohydride (Experiment 1)[a]

casein treatment[b]	wt gain,[c] g	food intake,[c] g	food efficiency,[c] wt gain/food intake
unmodified	34.0 ± 3.6[1]	153.8 ± 5.9[1,2,3]	0.22 ± 0.02[1]
glucose treated, − $NaCNBH_3$	35.6 ± 4.7[1]	162.6 ± 7.2[1,2]	0.22 ± 0.02[1]
fructose treated, − $NaCNBH_3$	35.6 ± 3.5[1]	172.6 ± 8.8[1]	0.21 ± 0.01[1,2]
lactose treated, − $NaCNBH_3$	33.0 ± 3.7[1]	158.8 ± 10.2[1,2]	0.21 ± 0.01[1,2]
glucose treated, + $NaCNBH_3$[d]	3.5 ± 2.7[3]	106.6 ± 9.8[4]	0.03 ± 0.03[3]
fructose treated, + $NaCNBH_3$[d]	21.6 ± 2.4[2]	131.0 ± 5.5[3]	0.16 ± 0.01[1,2]
lactose treated, + $NaCNBH_3$[d]	22.2 ± 4.4[2]	144.6 ± 8.1[2,3]	0.15 ± 0.03[2]

[a] Feeding period was 2 weeks. [b] Preparations of casein derivatives are described in the text. [c] Mean of five rats ± SEM. Values within a column without a common superscript number are significantly different ($P < 0.05$). [d] Extents of modification of lysyl groups were 80, 62, and 17% for glucose, fructose, and lactose treated casein, respectively.

Table V. Growth Response of Rats Fed Diets Supplying 10% Reductively Glucose-Derivatized Casein with or without 0.8% L-Lysine Hydrochloride Supplementation (Experiment 2)[a]

casein treatment	wt gain,[b] g	food intake,[b] g	food efficiency,[b] wt gain/food intake
unmodified	32.6 ± 3.4[1]	135.6 ± 7.2[1]	0.24 ± 0.01[1]
glucose-derivatized casein	14.8 ± 3.4[2]	110.0 ± 5.6[2]	0.13 ± 0.03[2]
glucose-derivatized casein plus 0.8% L-Lys·HCl	21.8 ± 1.9[2]	119.6 ± 4.2[1,2]	0.18 ± 0.01[1,2]

[a] Feeding period was 2 weeks. Glucose-derivatized casein showed 71% modification of lysyl residues. [b] Mean of five rats ± SEM. Values within a column without a common superscript number are significantly different ($P < 0.05$).

cose–casein group in experiment 2. Lysine supplementation promoted the average gain from 14.8 to 21.8 g/rat, even though this increase lacked statistical significance. The weight gains for these two groups were significantly lower than the 32.6 g/rat for the control group. Food intake and food efficiency of the unsupplemented group were significantly below the control values, but not different from the lysine-supplemented group.

DISCUSSION

A variety of chemical methods are available for the attachment of monosaccharides and oligosaccharides to proteins (Marshall, 1978). In this work the monosaccharides, glucose and fructose, and the disaccharide, lactose, were attached to the ε-amino groups of lysine in casein by reductive alkylation in the presence of cyanoborohydride. A pH study showed that the rate of coupling was lower at pH 7.0 than at pH 8.0 and 9.0. This pH effect has also been observed by Schwartz and Gray (1977). Borch et al. (1971) found that reductive alkylation in anhydrous methanol proceeded fastest at pH 7 where formation of Schiff base is favored, but the current findings and results reported by Schwartz and Gray suggest that Schiff base formation may not be rate limiting in the reductive alkylation by reducing sugars in aqueous solution.

No attempts were made to characterize the derivatized lysines after acid hydrolysis. Schwartz and Gray (1977) found that secondary amine linkages were formed upon reductive alkylation by disaccharides of the ε-amino groups of lysine in bovine serum albumin. They proved this by identifying and quantifying the amount of ε-*N*-1-(1-deoxyglucitol)lysine formed by complete acid hydrolysis of the conjugates. In addition, when the reductive alkylation reaction of lactose and α-*N*-acetyllysine was conducted using an excess of the disaccharide, small amounts of the tertiary amine, α-*N*-acetyl-ε-*N*-di[1-(1-deoxyactitol)]lysine, was also found.

The decrease in in vitro digestibility of the sugar–casein derivatives by α-chymotrypsin may be explained in terms of steric hindrance, substrate inhibition, and/or the formation of nonproductive complexes. Substrate inhibition has been observed in casein reductively alkylated with hydrophobic groups (Sen et al., 1979).

There appears to be a number of factors contributing to the lowered nutritive value of the sugar casein derivatives in the present study. In experiment 1, rats fed lactose-derivatized casein (17% modified) showed a similar degree of growth depression as those fed fructose-derivatized casein (62% modified). A more severe condition of diarrhea was noted in the lactose-derivatized casein group. In experiment 2 lysine supplementation of the diet containing glucose-derivatized casein showed some growth-promoting activity. However, normal growth was not obtained. These results suggest that the nutritive value of the sugar-derivatized caseins not only depends on the availability of lysine but also on the nature of the reducing sugars attached to lysine.

The metabolism and utilization of various sugar-derivatized amino acid model systems have been investigated extensively. Finot and Mauron (1969) synthesized ε-*N*-deoxyfructosyllysine as a model of the most stable browning compound. This compound appeared not to be utilized as a source of lysine by rats (Carpenter, 1973); a considerable proportion of the original compound was recovered in the urine and varying amounts in the feces. The varying amounts in the feces were explained by fermentation of fructosyllysine by the intestinal flora (Erbersdobler, 1975).

The antinutritive effects of fructose–tryptophan were found to be due to its competitive inhibition of the mucosal enzymes and its competition with tryptophan during absorption in the small intestine of rats (Lee and Chichester, 1975). Both in vitro and in vivo studies showed that the absorption of L-tryptophan was competitively inhibited by fructose–tryptophan. The inhibition of amino acid transport in rat small intestine by N$^{\epsilon}$-substituted L-lysine derivatives was also reported by Yasumoto et al. (1977). It may be possible that such an inhibitory mechanism exists in the absorption of sugar-derivatized lysines formed via reductive alkylation.

It is clear that the nutritional and metabolic consequences of feeding sugar-derivatized caseins in the present study are complex. Such factors as (1) incomplete proteolytic hydrolysis of proteins due to lysine modifications which in turn may lead to conformational and physical changes, (2) product inhibitory effects on mucosal enzymes and on the absorption of other nutrients, (3) intestinal disorders caused by floral fermentations of undigestible and unabsorbed substances, and (4) the unavailability of the derivatized amino acids should be carefully considered in future investigations.

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Covalent Attachment of Amino Acids to Casein. 1. Chemical Modification and Rates of in Vitro Enzymatic Hydrolysis of Derivatives

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Active *N*-hydroxysuccinimide esters of various *tert*-butyloxycarbonyl-L-amino acids were used to covalently attach amino acids to casein through isopeptide bonds. Tryptophan was used to determine the best conditions for the reaction; glycine, alanine, methionine, *N*-acetylmethionine, aspartic acid, and asparagine were also covalently linked to casein. In vitro rate studies performed with bovine α-chymotrypsin, bovine pancreatin, and rat bile pancreatic juice indicated that hydrolysis of the modified casein derivatives were lower than that of unmodified protein. The rates of decreased hydrolyses did not result from changes in solubility properties but was rather due to steric hindrance as well as conformational changes of the modified protein as shown by fluorescence and absorption spectra. The facile covalent attachment of amino acids to proteins appears to be a promising method for improving the biological value of food proteins.

During the past 15 years, a great deal of scientific interest has focused on problems of world protein supply and, as a consequence, the number of studies related to food proteins has been constantly increasing. However, recently it has become more evident that a large number of food proteins should have their biological quality improved in order to better meet increasing world protein needs. Improvement of food proteins not only includes an increase in the nutritional value of the protein but also better functional properties adaptable to new uses.

There have been many investigations on functional properties of food proteins and a review of the extensive documentation available suggests that this field of interest is still highly promising (Ryan, 1977; Schoen, 1977). A variety of physical (Huang and Rha, 1974), chemical (Frazen and Kinsella, 1976; Miller and Groninger, 1976; Feeney, 1977a), microbial (Whitaker, 1978; Beuchat, 1978), and enzymatic methods (Richardson, 1977; Whitaker, 1977) have been used to change the functional properties of food proteins.

Nutritive value of a food protein depends to a large extent on its content of essential amino acids. As a consequence, proteins from plants, single cells and other less conventional sources have limited nutritional value because of their low content of one or more essential amino acids. Because of the economical, political, and nutritional importance of this problem, a number of important studies have been done on food proteins to improve their nutritional value. The potentials for improving protein quality in plants by genetic means are quite promising and a few investigators have already been successful in improving seed protein quality by genetic and breeding practices (Mertz et al., 1964; Munck, 1972; Johnson and Mattern, 1978). Fortifications of foods and feeds with essential amino acids (Benevenga and Cieslak, 1978), supplementation of cereal proteins by oilseed proteins (Sarwar et al., 1978), or enzymatic protein degradation and resynthesis for protein improvement (Yamashita et al., 1976; Fujimaki et al., 1977) have also attracted a lot of attention. However, although the usefulness of such studies for improving protein quality has been demonstrated, none of these techniques are entirely satisfactory.

The aim of this work has been to investigate the feasibility of covalently attaching amino acids to casein, chosen as a model of food protein, in order to study the effects of newly linked hydrophobic or hydrophilic groups

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