ENZYMATIC PROTEOLYSIS OF SELECTED MILK PROTEINS AS AFFECTED BY HEAT TREATMENT AND OTHER AGENTS

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
GEORGE A. PURVIS
1969

This is to certify that the

thesis entitled

Enzymatic Proteolysis of Selected Milk Proteins as Affected by Heat Treatment and Other Agents.

presented by

George A. Purvis

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Food Science

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Major professor

Date January 23, 1969

O-169

ABSTRACT

ENZYMATIC PROTEOLYSIS OF SELECTED MILK PROTEINS AS AFFECTED BY HEAT TREATMENT AND OTHER AGENTS

By

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Milk proteins were studied during enzymatic proteolysis as model protein systems with and without heat treatment. The effect of selected components on enzymatic proteolysis of model systems was evaluated. Heat-induced protein interactions were considered as they influence enzymatic proteolysis.

This study was divided into three parts: pepsin-pancreatin hydrolysis of model proteins and assessment of amino acids and peptides liberated; evaluation of model proteins enzymatically hydrolyzed during gel filtration; and rate of proteolytic proton release monitored with a pH-stat.

Milk protein treatments included heat treatment (121 C for 30 min), sugars, chemical denaturing agents and addition of calcium and sodium salts.

An essential amino acid index was applied for interpretation of enzyme released amino acids and correlated with published biological data. Casein enzyme-released

amino acids were lowered 13% by heat treatment and 36% by heat treatment with added lactose. Enzyme liberated amino acids from α_s-casein were lowered 16% by heat treatment and 12% by heat with added lactose. k-Casein enzyme-freed amino acids were reduced 23% by heat treatment with added lactose. Amino acids freed in enzymatic digests of β-lactoglobulin were not changed by heat treatment.

Enzymatic hydrolysis of k-casein and β-lactoglobulin during gel filtration was 24% more extensive after heat treatment than before. The proteins were assessed for contents of ε-amino lysine before and after gel filtration proteolysis, and the data were ineffective indicators of susceptibility to proteolysis, possibly due to interference by terminal amino groups.

Rates of enzymatic proteolysis were estimated by pH-stat monitored proton release for proteins treated with chemical denaturing agents. Urea treatment of casein and β-lactoglobulin resulted in increased proteolysis rates; however heat treatment reduced proteolysis rates. Protein treatment with mercaptoethanol resulted in slightly reduced proteolysis rates for all proteins studied except α_s-casein. Performic acid oxidation of milk proteins increased rates of proteolysis, and rates were not significantly influenced by heat treatment.

Heat treatment at pH 5 reduced digestion rates of α_s-casein to immeasurable levels but increased k-casein

(26%) and β-lactoglobulin (20%). Heat treatment at pH 9 did not alter the rates of proteolysis for k-casein and β-lactoglobulin but reduced the rate for α_s-casein to 28% of the original level.

The model protein systems were used to provide an indication of enzymatic proteolysis of food proteins as affected by heat treatment and chemical denaturing agents. The observations derived can be used to describe similar treatment to other food protein systems. The amino acid release rate upon enzymatic proteolysis provides the basis for an index of protein digestibility. Measurement of rate of enzymatic proteolysis provides a potential method for rapid estimation of protein digestibility.

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Food Science

1969

ACKNOWLEDGMENTS

The author expresses his sincere appreciation to Dr. J. R. Brunner, Professor of Food Science, for his inspiration, counsel and patience during this study and for his assistance in the preparation of this manuscript.

Grateful acknowledgment is due Dr. B. S. Schweigert, Chairman of the Department of Food Science, for making arrangements so that this study could be made and for making the research facilities available. Acknowledgment is also due Miss Ursula Koch for analyses on the amino acid analyzer.

The author thanks Dr. R. A. Stewart, Director of Research, Gerber Products Company, for his encouragement. Grateful acknowledgment is due Gerber Products Company for financial support during this study.

The writer is grateful to his wife, Norma, for typing the drafts and for her understanding encouragement. The author also thanks his children: Amy, Beth, Tom and Meg for waiting.

TABLE OF CONTENTS

LIST OF TABLES

Table | Page

LIST OF FIGURES

INTRODUCTION

Milk proteins exist in nature as a complex mixture of micellar casein and whey proteins. Individual milk proteins have characteristics which have been established by physical and chemical studies. The individual milk proteins derived have been used as model protein systems for the study of enzymatic proteolysis with and without heat treatment in this study. An effort has been made to evaluate some of the components of foods on the model protein systems as they affect enzymatic hydrolysis. Protein interactions induced by heat have been reported as they are measured by physical behavior. The interactions thus described have been further examined relative to the influence they may have on proteolytic behavior.

The enzymatic breakdown of proteins has been related to digestion of proteins during the digestive process. The use of model protein systems to further describe the breakdown of proteins may provide a further insight into the behavior of proteins upon digestion.

Measurement of protein quality as the ability to support growth has been related to amino acid content, specific amino acid availability and amino acid release upon exposure to proteolytic enzymes. The rate of

proteolysis of a protein may provide another means to attain an indication of protein quality.

This study was conducted to examine the behavior of model milk protein systems during enzymatic proteolysis. The observations thus derived can be related to the treatments encountered in food processing operations. The behavior of model proteins upon hydrolysis can also be associated with the behavior during digestive processes.

LITERATURE REVIEW

The primary importance of enzymatic proteolysis is the specific set of functions as performed in the digestive process. The breakdown of dietary proteins involves degradation by enzymatic cleavage to yield residues to be absorbed by the intestinal mucosa. The proteins ingested are subjected to proteolysis by successive attack by first pepsin at a relatively low pH, then by the proteolytic enzymes secreted by the pancreas at higher pH. The conditions for proteolysis are optimal for each enzyme. The optimal pH for pepsin is 1.8 compared to 7-8 for trypsin and pancreatin. The temperature optimum for each of the proteolytic enzymes considered is 37 C.

The order of hydrolysis must be taken into account. Initially pepsin acts in the gastric juice at pH 1.8-2, followed by pancreatic enzymes in the intestine at pH 7-8. The systems must be considered in the order of their place in the digestive process.

The principle enzyme of gastric juice is pepsin (3.4.4.1) which has a pH optimum of 1.8 as described by Bovey and Yanari (1960). Further proteolysis is accomplished after passage to the intestine and adjustment of the intestinal pH to between 7 and 8, by trypsin (3.4.4.4)

and chymotrypsin (3.4.4.5). The enzymes trypsin and chymotrypsin are so closely related in their behavior that they may be discussed together as Desnuelle (1960) has suggested. The characteristic action of trypsin and chymotrypsin includes not only cleavage of peptide linkages, but ester linkages as well, probably by attack on the nucleophilic centers and serine moieties as described by Cunningham (1957). The mechanism of action for trypsin has been suggested by Graae and Rasmussen (1961) to be identical for proteins of different structures. Smith and Hill (1960) proposed that leucine aminopeptidase (3.4.1.1), sometimes referred to by its trivial name, erepsin, should be given more active consideration in physiologic proteolysis.

The specificity of each of the enzymes involved in physiologic digestion is unique and has been summarized by Hill (1965). Pepsin is least specific of the proteolytic enzymes involved, cleaving amino or carboxyl groups of phenylalanine, tryosine, glutamic acid, cystine and cysteine. The specificity of rennin (3.4.4.3) and cathepsin (3.4.4.9) is similar to pepsin. Trypsin peptide specificity is restricted to peptide bonds formed by carboxyl groups of lysine and arginine. Chymotrypsin cleaves at the carboxyl bonds of tyrosine, phenylalanine and tryptophan. Leucine aminopeptidase cleaves peptide bonds adjacent to the α-amino group but shows preferential treatment to leucine residues. Specificity for each of

the enzymes is also influenced by the isomeric form, with the L-form favorably attacked as described by Rapp et al. (1966).

Proteolysis during digestion is specific not only to individual proteins but specificity is changed by the condition of the individual proteins. Structural differences in native proteins, specifically plasma albumin and γ-globulin, have been observed by Epstein and Possick (1961) to influence their susceptibility to enzymatic cleavage. They attributed these differences to stresses involved in tertiary linkages between polypeptide chains. Allison (1964) pointed out that each native protein is unique in its proteolysis in much the same way that behavioral reactions are unique and that proteolysis is as dependent on structure as is behavior.

Donoso et al. (1962) proposed that changes occurred in structural characteristics of pork protein as a result of extensive heating. These included formation of aldehyde condensation products and reduction of disulfide cross-linkage reaction products to sulfhydryl groups. Neither of the protein treatments exhibiting these types of interactions were available to enzymatic cleavage.

The products of proteolysis, amino acids and "oligopeptides" (i.e., peptides with 2-4 amino acids) have been studied as important determinants of rate and extent of intestinal absorption by a group of British researchers;

Matthews, Crampton and Lis (1968), Matthews, Craft and Crampton (1968), and Hamilton et al. (1968). They postulate that protein degradation products may be most favorably absorbed as "oligopeptides" although hydrolysis to amino acids must occur prior to entry into the blood. The transport of dipeptides (methionyl-methionine, methionyl-glycine, glycyl-methionine) was more rapid than component amino acids. The transport rate for each dipeptide studied was unique. This may partially explain the distinctly greater utilization of pathways alternate to the glycolytic scheme observed by Ahrens and Wilson (1966) in rats fed casein as opposed to rats fed an amino acid mixture simulating casein.

The release rate and total content of amino acids was comprehensively examined by Block and Mitchell (1946). They observed that the value for growth support is dependent not only on total content of amino acids but on relative proportion of these amino acids.

The rate of proteolysis of several proteins from animal and plant sources was studied in white rats by Zebrowski (1968). Casein emptied from the stomach much more rapidly than did heated soya protein. The intestines contained more soluble nitrogen when the rats were fed casein diets as compared to heated soya protein diets. The intestine contained extremely large amounts of protein nitrogen when rats were fed raw soya protein relative to either heated soya or casein.

The influence of heat treatment of protein on rate and extent of hydrolysis has been related to a number of considerations including structure of the protein and reactions of individual groups within the protein molecule. Kakade and Evans (1966a) credited denaturation of navy bean protein for improved proteolysis as measured by lysine availability. They separated the effect of a heat labile trypsin inhibitor in a further study (Kakade and Evans, 1966b) as an independent consideration.

The extent of heating and protein concentration were related to the rate of disappearance of food from the digestive tract by Rice and Beuk (1953). They noted that heating of casein for 8 hr at 100 C did not reduce the growth-promoting properties of casein, however toasting did cause an impairment. Heat treatment of evaporated milk for a period of 3 hr was required at 120 C to result in measurable depression of rat growth.

The loss of "nutritive efficiency" after heating milk was reported by McCullom and Davis in 1915. The loss was attributed to "sulfur" due to the characteristic sulfur odor upon heating. The observation that destructive changes take place has been developed from several aspects by numerous studies.

The most striking results for milk protein alone have been accomplished with milk powder after autoclaving. Kraft and Morgan (1951) fed rats milk powder heated for

15 to 25 min at 120 C. Rat growth was depressed by heat treatment to 50% of the rate when rats were fed control diets. The lack of rat growth could be reversed by addition of lysine. In an experiment with weanling dogs for a 120 day period with the same diets there was no observable difference in growth rate or nitrogen balance. Eldred and Rodney (1946) performed a study *in vitro* with heat treatment of dry casein at 150 C for 70 min. Stepwise hydrolyses were performed with pepsin, trypsin and pancreatin; and lysine was determined manometrically with lysine decarboxylase. Digestion rates measured by Van Slyke α-amino nitrogen did not differ significantly, however lysine contents were significantly lower than observed with unheated casein. Acid hydrolysates of heated and unheated samples were not measurably different in their amino-acid content. Hankes *et al.* (1948) made a similar observation that autoclave heating of casein for as long as 15 hr did not reduce the content of total amino acids but did reduce *in vitro* digestibility by 25 to 47%. Losses in lysine, methionine and tryptophan in commercially processed products were reported by Mauron *et al.* (1951). The only products they examined which reflected significant losses were roller-dried milk powder (23 to 62%) and evaporated milk (11%). Spray-dried milk powder, sweetened condensed milk and boiled milk losses were minimal. In contrast, Fricker (1964) fed rats through five

generations raw milk and "Uperized" (flash heated) milk heated to 150 C for 2.4 sec. He was unable to observe any differences in rate of growth, histological development or reproductive ability.

Heat treatment of milk proteins with sugars has been examined extensively from the standpoint of susceptibility to hydrolysis. Patton et al. (1948) were able to demonstrate decreases of 15 to 27% in lysine, arginine, and tryptophan in 5% casein-glucose solutions heated for 24 hr at 95.5 C. McInroy et al. (1948) demonstrated the relative effects of added dextrose and protein concentration after heat treatment at 121 C for 2 hr. They reported that rats lost weight with casein and dextrose autoclaved; however growth rates were 79% of the control when casein was autoclaved alone. Mixtures of casein with dextrose at 50% moisture were examined by Lowry and Thiessen (1950) with in vitro enzymatic digestion. Pepsin was effective in hydrolysis of the casein-dextrose complexes while trypsin was almost totally ineffective as measured by α-amino nitrogen release. They suggested the formation of ester linkages by casein molecules which were susceptible to pepsin cleavage but not to trypsin. Lowe et al. (1964) demonstrated significant growth advantage in infants fed a formula sterilized at 146 C for 6 sec when compared to conventional autoclave processes. The pertinent measurements included weight gain per energy intake as well as

total growth measurement. Differences in nitrogen balance and serum proteins were not discerned by Fomon and Owen (1962) for infant formulas fed with and without autoclaving.

Milk proteins must be distinguished as to their presence in milk or as derived from milk. The native state of the individual proteins is in the form of a complex as a stable colloidal suspension. Casein, as described by Morr (1967), consists of loosely packed calclum caseinate complex units joined by calcium and calcium phosphate-citrate linkages. Casein has been defined by Thompson et al. (1965) as a heterogeneous group of phospho-proteins precipitated from skim milk at pH 4.6 and 20 C. The complex units or micelles in casein are stabilized by interaction of the casein fractions as described by Waugh (1961). The k-casein fraction is believed to function as a "protective colloid" for micellar casein (Swaisgood et al., 1964). Thompson et al. (1965) have defined k-casein as a phospho-glyco-protein capable of stabilizing α_s-casein against precipitation by calcium. The proteins remaining after casein has been removed from skim milk comprise whey proteins or milk serum proteins as designated by Gordon and Whittier (1965). The protein fractionated from milk designated as α_s-casein is a calcium sensitive, disordered protein without intermolecular disulfide bonds as described by McKenzie (1967). The

behavior of α_s-casein is advantageous in that it is susceptible to digestion particularly by the newborn because of its disordered configuration. In contrast, k-casein has been described by Swaisgood et al. (1964) as an ordered glyco-protein with intramolecular disulfide bonds determining a specific tertiary configuration. The carbohydrate component of k-casein is sialic acid. Waugh (1961) concluded that all the cystine in casein existed in the k-casein fraction. The k-casein fraction is sensitive to specific attack by rennet. The "caseino-glycopeptide" formed by heat treatment was reported by Alais et al. (1967) to be similar to the rennet hydrolysis product.

The milk whey protein β-lactoglobulin is a globular protein containing disulfide linkages and sulfhydryl groups susceptible to reversible disulfide interchange as reported by Morr (1967). β-lactoglobulin was considered by early workers to have external secondary structure due to its ability to form crystals. More recent information compiled by Tanford et al. (1962) indicates that the native structure is compact and that refolding after denaturation results in a large number of α-helices. Tanford et al. (1967) have further observed that specific and different denatured states are produced by explicit denaturing agents. McKenzie (1967) has concluded that β-lactoglobulin exhibits a high degree of change in secondary structure depending on environmental conditions.

A proteolytic enzyme was reported in casein by Warner and Polis in 1945 which decreased casein solubility and increased trichloroacetic acid soluble nitrogenous products. Harper et al. (1960) reported that raw milk usually but not always contains a small amount of a protease which releases tyrosine. Zittle (1963) observed the association of milk protease with the k-casein fraction. Trypsin inhibitor was reported by Kiermeer and Semper (1960) in cow's milk. The presence of both a protease and inhibitor has been suggested by Shahani (1966) as a reason for inability to establish quantities or activities for either component.

The ordered form of a native protein may be disrupted as described by Kauzman (1956) to modify the primary, secondary, or tertiary structure. The properties of a native protein may be sensitive to change by heat, light, pressure or chemical reagents. Chemical denaturing agents have been examined and specific changes attributed to them. Performic acid has been utilized by Hirs (1956) for oxidation of the sulfur moieties of sulfur-containing amino acids. Proteins oxidized with performic acid were approximately one-tenth as susceptible to tryptic digest as were the original proteins. Mercaptoethanol has been utilized for reversible disulfide interchange. Acid and base binding groups were released by denaturation of ovalbumin with guanadine by Harrington (1955).

Electrophoretic changes in casein were observed by Tamauchi and Tsugo (1961) as a result of heating skim milk. Heat treatment at 120 C for 30 min resulted in severely decreased contents of α_s- and β-caseins. The changes were probably due to complexes of the type described by Trautman and Swanson (1959). The complexes they observed between α-casein components and β-lactoglobulin could be prevented by sulfhydryl blocking agents. Morr and Josephson (1968) reported that whey protein aggregation was influenced by thiol-disulfide group reactions and calcium concentration.

The degree and severity of heat denaturation can be altered by a number of factors; pH, ionic strength, ultraviolet treatment and concentration; as described by Joly (1965). The stabilization against heat denaturation by anions of fatty acids was reported by Boyer et al. in 1946. Epstein and Possick (1961) suggested that fatty acid anions reduce denaturation to cause a reduction in susceptibility to enzymatic proteolysis. Protein denaturation may occur by enzymatic action when hydrolysis does not occur as observed by Lundgren (1941) with thyroglobulin. Allosteric effects may in part account for reversible denaturation observed in proteolytic enzymes (Gerhart and Pardee, 1962).

The influence of denaturation on susceptibility to proteolysis is unique to the substrate protein.

Linderstrøm-Lang et al. (1938) cited an example of enhanced tryptic proteolysis of β-lactoglobulin when the temperature was adjusted to induce the denatured form of the protein. Ledford et al. (1968) reported that α_s-casein was hydrolyzed before whole casein or β-casein by rennin. In studies with k-casein, Zittle (1961) noted that in combination with heat, chymotrypsin destroyed the stabilizing property of k-casein for α_s-casein in the presence of calcium ions.

Measurement of protein quality has traditionally been made by growth of animals, usually rats, under carefully defined conditions. Expression of protein quality has been as Protein Efficiency Ratio (PER) or gain in body weight divided by amount of protein consumed (Campbell, 1963). Another method for expression described by Miller (1963) is Net Protein Utilization (NPU) or carcass nitrogen with test protein compared to control protein. Many refinements and modifications of these basic methods have been used for specific purposes: AOAC, 1965; Braham et al., 1959; Summers and Fisher, 1961; Derse, 1962.

The relative amino acid contents have also been used as measurements of protein quality, since the nutritive value of dietary protein depends on the pattern and quantity of essential amino acids. The two most common methods for expression of amino acid values have been the Chemical Score suggested by Mitchell and Block (1946) and

the Essential Amino Acid Index proposed by Oser (1951). Both techniques establish an index based on the amino acid composition of protein relative to the requirements of the rat. Comparison may also be to a reference pattern such as that suggested by the FAO (1961). Advantages of speed can be attained with chemical methods, however losses during hydrolysis as reported by Patton (1950) and variations in digestibility and availability as noted by Akeson and Stahmann (1964) diminish their value.

A number of enzymatic digest methods for protein evaluation have been developed to overcome the disadvantages of animal studies and chemical methods; time, expense and lack of accuracy. An early method was proposed by Sheffner et al. (1956) involving a pepsin digest followed by microbiological examination of residual protein and released essential amino acids. Mauron et al. (1955) utilized sequential digestion with pepsin and pancreatin in a dialysis sac. Measurements of the amino acids tryptophan, tyrosine, methionine and lysine were accomplished in the dialysate using manometric techniques. Ford and Salter (1966) used a similar hydrolysis, removing the amino acids and small peptides on Sephadex gel (G-10). The retained residues were quantitated microbiologically. A stepwise hydrolysis method was reported by Frangne and Adrian (1967) utilizing pepsin, pancreatin and erypsin, sequentially applied. Unhydrolyzed proteins were removed

by alcohol and heat treatment. Lysine and methionine were estimated in the filtrate by microbiological methods. The technique suggested by Akeson and Stahmann (1964) involved hydrolysis with pepsin then pancreatin followed by analysis for amino acids of the picric acid supernatant by means of an automatic amino acid analyzer. Each of the enzymatic degradation techniques described here provided an accurate index of protein quality as related to animal growth measurements.

Chemical indices of protein quality have been directed to lysine studies. Selection of lysine was based on observations by Lea and Hannan (1950) and Henry and Kon (1950) that availability of lysine was paramount to chick growth on the protein under consideration. Donoso et al. (1962) suggested that cross linkage reactions as well as condensation reactions were responsible for loss of lysine in heated systems. The reaction of the Sanger reagent, 1-fluoro-2, 4-dinitrobenzene (FDNB), with protein to form epsilon-2, 4-dinitrophenyl lysine was applied by Carpenter (1960) to measure the availability of lysine in aminal proteins. Evaluations of the technique have been performed independently by several groups. Roach et al. (1967) noted that differences in heat treatment could be measured quantitatively. Bujard et al. (1967) observed values for the FDNB method that fell between the total lysine contents as determined enzymatically and those

determined by hydrolysis. From their studies with animals, Boctor and Harper (1968) concluded that the FDNB method was not suitable for estimation of lysine in heat-treated foods. Some of the lysine reported as available with FDNB was a part of the undigestible residue in the feces of rats. Either a combination of measurements or a more precise measurement of amino acid availability is therefore indicated.

Commentary and Objectives

Protein systems and food protein mixtures have been examined in many studies relative to their susceptibility to enzymatic proteolysis. The characteristics of many individual proteins have been reported in terms of physical and chemical properties. The functions of proteolytic enzymes have been separately defined, usually with single substrates. Specific relationships between protein systems, component protein fractions and protein treatments are, however, not well defined in terms of enzymatic hydrolysis. Animal studies have been used to indicate total protein digestion; however *in vitro* techniques are more definitive because stepwise observations can be made.

The objectives of this study include an evaluation of enzymatic proteolysis of milk proteins treated as model protein systems. The model protein systems were selected to be representative of food proteins. Treatments chosen are commonly encountered in processing of food

proteins. Additional selected chemical treatments were applied to evaluate the effect on enzymatic proteolysis. One of the purposes of this study is to apply the amino acid release and proteolysis rate data to a method for accurate assessment of protein digestibility. Another purpose is to describe differences in behavior upon proteolysis of model milk protein systems and to relate them to previously described physical and chemical protein differences. A third purpose is to examine existing methods for protein evaluation as applied to model protein systems.

EXPERIMENTAL

Apparatus and Equipment

The milk used for preparation of milk protein fractions was collected in five-gallon stainless steel cans and separated with a DeLaval Model 9 disc-type separator. A Beckman expanded scale pH meter with glass electrode was used to measure pH. Low speed room temperature centrifugations were performed with an International Model U centrifuge. Intermediate speed and refrigerated centrifugations were performed with a Sorvall RC-2B refrigerated centrifuge.

The eluate from gel filtration columns was monitored at 280 nm with an ISCO UA-5 recording monitor manufactured by Instrument Specialties Company.

Laboratory weightings were made utilizing a top-loading, direct-reading Mettler Type K-7 balance. Analytical weighings were made with a Mettler H-15 analytical balance.

Amino acid analyses were performed with a Beckman-Spinco Model 120C amino acid analyzer.

A Coleman Model 14 Universal Spectrophotometer was used for all spectrophotometric examinations.

Proton release during enzymatic proteolysis was measured with a Sargent Recording pH-Stat.

Chemicals and Materials

The principle chemicals used and their suppliers are given below. Glucose and lactose were Baker Analyzed and obtained from J. T. Baker Chemical Company. Galactose was Mann Assayed from Mann Research Laboratories, Inc. Bio-Gel P-2 and a 1% solution of dimethyldichlorosilane in benzene were obtained from Bio-Rad Laboratories. The anion exchange resin, Dowex 2-X 8, was purchased from J. T. Baker Chemical Co., as were 2-mercaptoethanol and 1-fluoro-2, 4-dinitrobenzene. The N-dinitrophenyl-epsilon-L-lysine monohydrochloride used as a standard was obtained from Mann Research Laboratories.

The trypsin was 2X recrystallized purchased from Worthington Biochemical Corporation. Pepsin utilized was 3X recrystallized from General Biochemicals. Pancreatin was "B" Grade purchased from Calbiochem.

The other chemicals used in this study were of reagent grade.

Preparative Procedures for Milk Protein Fractions

Milk used in this study was obtained from the Michigan State University dairy herd which consisted of Holstein, Jersey and Brown Swiss cows. All milk was

obtained immediately after milking and separated while still warm.

Preparation of Whole Casein

Whole casein was prepared from fresh skim milk by precipitation at pH of 4.5 at 25 C. The precipitated whole casein was filtered from the supernatant with double cheesecloth and drained for 15 minutes. The casein was redissolved at 3% in deionized water by adjustment of pH to 7 with 0.1 N NaOH. The sodium caseinate is referred to as casein. The casein solution was stored at -20 C in approximately 100 g quantities for subsequent use.

Preparation of α_s-casein

The method of preparation for α_s-casein was suggested by Zittle and Custer (1963). Approximately 350 g of frozen whole casein were dissolved in 1 liter of 6.6 M urea. The solution was acidified with 200 ml of 7 N sulfuric acid and 2 liters deionized water added. The resulting pH was 1.4 to 1.5. A flocculent precipitate which formed during a 2 hr period was removed by centrifugation. The centrifugal supernatant was retained for preparation of k-casein. The aforementioned precipitate was dissolved by adjustment of the pH to 7.2 with 0.1 N NaOH. The resulting clear solution was added to an equal volume of 95% ethanol until maximum precipitation was achieved. The precipitate was removed by centrifugation and discarded. The supernatant

was acidified to pH 5.0 with 3.0 N HCl. The precipitate formed in this operation was separated by centrifugation and dissolved at pH 7.5 with 0.1 N sodium hydroxide. The resulting α_s-casein solution was dialyzed against deionized water and pervaporated to a concentration of approximately 10 mg per ml.

Preparation of k-casein

The method proposed by Zittle and Custer (1963) also was used for k-casein. The supernatant from the acid-urea fractionation of α_s-casein was treated with 132 g ammonium sulfate per liter. The precipitate formed was removed by centrifugation, suspended in deionized water and dissolved by adjustment of the pH to 7.5 with 1.0 N sodium hydroxide. The solution, containing principally k-casein, was dialyzed against deionized water and pervaporated to a concentration of approximately 10 mg protein per ml.

Preparation of β-lactoglobulin

The preparation method for β-lactoglobulin was that suggested by Fox et al. (1967). The acid whey was recovered from casein preparation at pH 4.5. Trichloroacetic acid was added to the solution at a rate of 34.2 g per liter. The precipitate was removed by centrifugation at room temperature and discarded. The clear supernatant was concentrated by pervaporation to one-tenth the

original volume. Saturated ammonium sulfate was added in an amount to yield an 0.4 saturated solution with reference to ammonium sulfate. The mixture was allowed to stand for 12 hr at room temperature, and the precipitate was removed by centrifugation. The precipitate was suspended in saturated ammonium sulfate and dialyzed against deionized water at 4 C. The protein concentration was adjusted by pervaporation to approximately 10 mg protein per ml. A summary of milk protein preparation procedures is presented in Figure 1.

Acrylamide Gel Electrophoresis

The electrophoretic homogeneity of milk protein fractions was established with the continuous acrylamide gel electrophoresis system used by Peterson (1963). The buffer system was a tris-borate buffer with disodium ethylenediamine-tetraacetic acid (disodium EDTA). Stock buffer was diluted 1:9 for addition to buffer tanks. The acrylamide gel for one 250 ml gel bed was prepared by adding 27.5 ml stock buffer, 17.5 g Cyanogum 41 and 62.5 g urea to 150 ml glass distilled water. Immediately before pouring the liquid gel into the gel bed, 0.5 ml β-amino propionitrile and 0.3 g ammonium persulfate were added to the solution. After pouring, the gel was polymerized in a nitrogen atmosphere for 20-30 minutes and covered with saran wrap to minimize evaporation. The diluted tris-borate buffer was poured into the buffer tanks

to complete the system. Samples were introduced into pre-formed slots in amounts equivalent to 1-2 mg protein. One drop of a bromo phenol blue solution was introduced into one of the slots to act as an indicator since bromo phenol blue migrates with the most rapidly moving proteins. Platinum electrodes were inserted in the buffer tanks. Current was applied with a Heathkit JP-32 power supply, with an initial voltage of 100 volts, gradually increased to 250 volts after 15 minutes with a current of 75 ma. Electrophoresis was continued for 5 hr, at which time the dyd marker had moved within 2 cm of the end of the gel bed. When electorphoresis was completed, the gel was removed from the gel bed and stained with a dye solution containing 250 ml water, 250 ml methanol, 50 ml glacial acetic acid, and 5 g Amido Black. After a staining period of 30-40 min, the gel was electrolytically destained in a 7% acetic acid solution. The destained gel was then wrapped in saran wrap to prevent evaporation. A single α_s-casein band moved near the dye marker, β-lactoglobulin moved in a single band directly behind, and k-casein moved in a diffuse single band approximately 5 cm from the origin.

Preparative Procedures for Treated Milk Protein Fractions

The milk protein fractions were treated with heat in a manner consistent with normal processing practice. The

proteins were heated alone as well as with reducing sugars. Chemical denaturing agents intended to modify the configuration of the proteins were employed in other experiments.

Heat Treatment of Milk Proteins

The milk protein fractions were prepared, as previously described in concentrations adjusted to 10 mg protein per ml. The solutions were placed in 250 ml Erlenmeyer flasks and covered with aluminum foil. The flasks were subjected to autoclave treatment at 15 lb pressure for a period of 30 min in an Amsco Cyclomatic Sterilizer. After sterilization, the samples were immediately cooled and stored at 4 C until use.

Heat Treatment of Milk Proteins with Added Lactose

Milk protein fractions adjusted to protein concentrations of 10 mg per ml were made to 0.06 M lactose. Heat treatment was identical to that for heated proteins, in the same autoclave whenever possible. The pH of samples was adjusted to 7 prior to heating in all cases unless otherwise indicated.

Samples designated as heated milk proteins and milk proteins heated in the presence of lactose were the basic samples used in all evaluations of enzymatic hydrolysis; pepsin and pancreatin, gel filtration and measurement of proton release. All other variables were evaluated only

by monitoring of proton release during proteolysis by pH-stat. Observations were made with control samples, samples after heating and after heat treatment in the presence of lactose.

Variable samples were prepared from milk protein fractions for enzymatic proteolysis. Treatments included denaturation with selective denaturing agents; 2-mercaptoethanol, performic acid, urea, pH adjustment, and pepsin digest prior to tryptic proteolysis. The samples were treated with salts of the cations of sodium and calcium. Protein concentrations were adjusted according to the number of cleavage sites in the individual protein preparations to provide equal concentrations with respect to number of trypsin cleavage sites (lysine and arginine residues). Preparations were made of proteins with selected sugars for measurement of proton release upon hydrolysis.

Pepsin Treatment

Milk protein fractions, adjusted to a concentration of 10 mg protein per ml, were subjected to pepsin treatment according to the procedure suggested by Rick (1963). Pepsin was added in quantities equivalent to 1 mg pepsin per 100 mg protein. Proteolysis was conducted in a water bath at 37 C for 3 hr. The pH was then adjusted to 8.0 and tryptic proteolysis evaluated by monitoring the protons released with a pH-stat.

Treatment with Isotonic Saline

Milk protein fractions were treated by the addition of sodium chloride to yield a solution 0.15 M in sodium chloride.

Treatment with Calcium Ions

Milk protein fractions were treated by the addition of calcium chloride to yield a solution 0.3 M in calcium.

pH Adjustment

Milk protein fractions were heated at pH 5 and pH 9. The other samples were adjusted to pH 7 prior to heating. The pH adjusted samples were divided after heating and one portion treated with pepsin prior to evaluation of their susceptibility to proteolysis by pH-stat monitoring.

Equal Cleavage Sites

Concentrations of milk protein fractions were adjusted to establish an equal number of lysine and arginine residues per unit volume for each protein. The amino acid content of casein assumed for these calculations was that of Block and Weiss (1956), with the number of lysine and arginine residues 26.2 per molecular weight. The molecular weight of α_s-casein used was 28,000 although the range for genetic variants may be from 28-30,000, as discussed by Gordon et al. (1965). The number of lysine and arginine residues per monomer (24.3) was also reported by Gordon et al. (1965). The molecular weight for k-casein

(28,000) and number of lysine and arginine residues (18.0) of Swaisgood et al. (1964) were utilized. The values reported by Kalan et al. (1964) for β-lactoglobulin as monomer molecular weight (18,000) and number of lysine and arginine residues per monomer (18.0) were used.

Treatment with Sugars

Milk protein fractions adjusted to a concentration of 10 mg per ml were treated with selected sugars. Lactose was added at a rate of 20 mg per ml to yield a concentration of 0.06 M in lactose. Glucose and galactose were added to separate solutions at a rate of 10 mg per ml to yield a concentration of 0.06 M in glucose and galactose respectively. Glucose and galactose were added together to the milk protein fractions at rates of 10 mg per ml each to yield a concentration 0.06 M in each glucose and galactose.

Lactose represents a reducing disaccharide native to milk. Glucose and galactose are reducing monosaccharides. Glucose and galactose are the monosaccharide components of lactose and were included for evaluation of the effect of molar concentration.

The protein fractions prepared with sugars were divided with one portion heated and the other retained as a control. Protein-sugar preparations thus treated were examined for behavior upon proteolysis by monitoring of proton release using the pH-stat.

Treatment with Denaturing Agents

Performic Acid

The protein fractions were oxidized with performic acid using the procedure suggested by Hirs (1956). Performic acid was prepared by addition of 0.5 ml of 30% hydrogen peroxide to 4.5 ml 88% formic acid at 50 C. The performic acid so prepared was added to protein fractions in a volume of 0.8 ml performic acid to 10 ml sample solution. The reaction mixture was held at 50 C for 15 min and the performic acid was removed by passage over a Bio-Gel P-2 desalting gel. Protein recovery was monitored by measuring absorption at 280 nm. Aliquots of performic acid oxidized samples were further treated with heat and heat in the presence of lactose as previously described.

2-Mercaptoethanol

The disulfide linkages of protein fractions previously described were disrupted with 2-mercaptoethanol as suggested by Anfinsen and Haber (1961). 2-Mercaptoethanol was added to the protein fractions in amounts of 0.1 ml per 10 ml sample at 1 mg protein per ml in 0.1 N acetic acid. The reaction was allowed to proceed for 30 min in a nitrogen atmosphere. Freshly boiled deionized water was used in preparation of all reagents and for dilutions. Removal of 2-mercapthethanol was accomplished by passage over a Bio-Gel P-2 desalting gel with protein

recovery monitored at 280 nm. Aliquots of 2-mercaptoethanol-treated samples were further treated with heat and heat in the presence of lactose as previously described.

Eight Molar Urea

The milk protein preparations of casein and β-lactoglobulin were treated with 8 M urea to dissociate the secondary structural characteristics. Urea in amounts of 4.8 g per 10 ml was added to the preparations of casein and β-lactoglobulin adjusted to 1 mg protein per ml and dissolved by stirring with a magnetic stirrer. The solution was allowed to stand at room temperature for 30 min. The urea was removed by passage over a Bio-Gel P-2 desalting gel with monitoring of protein elution at 280 nm.

Chemical Analyses

Nitrogen

Micro-Kjeldahl nitrogen analyses were performed in duplicate. An apparatus with round-bottom flasks and ground glass joints was used. The digestion mixture was concentrated sulfuric acid (500 ml) to which 5.0 g SeO_2 and 5.0 g $CuSo_4 \cdot 5H_2O$ were added. The mixture was vigorously shaken before each usage. Ten to fifty mg of protein were digested with 4 ml of the digestion mixture for 1 hr or more. After cooling, 1 ml of 30% H_2O_2 was added and digestion continued for 1 hr. The digestion

flasks were rinsed with 10 ml of deionized water after cooling and connected to the distillation apparatus. Approximately 25 ml of a 40% sodium hydroxide solution was added to neutralize the digest. The released ammonia was distilled into a flask containing 15 ml of a 4% boric acid solution to which 4 to 5 drops of indicator had been added. The indicator contained 400 mg bromcresol green and 40 ml methyl red in 100 ml of 95% ethanol. Distillation was terminated when a total volume of at least 60 ml was present in the receiving flask. The ammonia was titrated with 0.1036 N hydrochloric acid. Tris(hydroxymethyl)aminomethane (Sigma 121) was used as the primary standard for determination of the normality of the hydrochloric acid.

Total Protein

Total protein was determined using the Folin reaction as described by Lowry et al. (1951). A reagent solution was prepared containing 50 ml 0.1 N sodium hydroxide with 2% Na_2CO_3 and 1 ml of a sodium tartrate solution with 0.5% $CuSo_4 \cdot 5H_2O$. One ml of the reagent was added to a protein solution containing 10 to 100 mg protein. The mixture was thoroughly mixed and allowed to stand for 10 min at room temperature. Folin-Ciocalteu reagent, diluted to 1 N in acid, was added in an amount of 0.1 ml. The reaction mixture was allowed to stand 30 min at room temperature. Optical Density (OD) was

measured at 700 nm. The relationship between protein concentration and OD was linear within the range 100-400 µg per ml. The linear equations were the same for casein, α_s-casein and k-casein, however a different relationship existed for β-lactoglobulin. The equations were as follows:

Caseins--Optical Density = 0.45 (µg Prot/ml) + 0.101

β-Lactoglobulin--Optical Density = 0.59 (µg Prot/ml) + 0.0876

(Kjeldahl nitrogen x 6.38 was used as a reference standard for all milk protein cnalyses.)

Trichloroacetic Acid Soluble Protein

The protein in trichloroacetic acid supernatants was determined by the McDonald and Chen (1965) modification of the method of Lowry et al. (1951) previously described. The modification involved preparation of a reaction mixture containing sufficient NaOH to neutralize the trichloroacetic acid and acid in the Folin-Ciocalteu phenol reagent. Standard curves were prepared and were identical to those for total protein.

ε-Amino Lysine

The method suggested by Carpenter (1960) for available lysine (ε-amino lysine) was adapted for heated samples and selected hydrolysates. The principle of the procedure was to convert lysine residues with reactive ε-NH_2 groups to yellow ε-DNP lysine by treatment with 1-fluoro-2, 4-dinitrobenzene (FDNB), followed by acid hydrolysis. Duplicate samples were prepared containing 30-50 mg protein in flat-bottom boiling flasks with ground glass fittings. To each of the flasks was added 8 ml of 8% (w/v) $NaHCO_3$ followed by 12 ml ethanol containing 0.3 ml FDNB. The flasks were covered and shaken gently on a mechanical shaker for 2 hr. The flasks were held in a boiling water bath until there was no further effervescence upon swirling. The flasks were cooled, 24 ml 8.1 N hydrochloric acid added, and gently refluxed for 16 hr on a hot plate with water cooled condensors. The flasks were cooled and placed in an ice bath for 1 hr, then filtered through Whatman No. 41 filter paper for removal of unreacted FDNB. The filtrate was made to 100 ml with 1 N hydrochloric acid. Interferring substances to colorimetric determination were removed by extraction with 50 ml ethyl ether. Optical density of the solution was determined at 435 nm with a Coleman Model 14 Spectrophotometer.

The standard curve was prepared using N-dinitrophenyl-epsilon-L-lysine monohydrochloride (ε-DNP-Lysine) in the

range 20 to 200 μg per 10 ml aliquot. The equation for the standard curve was:

$$\text{Optical Density} = 0.58\ (\mu g\ \varepsilon\text{-DNP-Lysine}/10\ ml) + 0.012$$

(Results were expressed as mg ε-DNP-Lysine per 100 g protein.)

Amino Acids--Acid Hydrolysates

Amino acid analyses were performed on hydrochloric acid hydrolysates of the proteins using a Beckman Amino Acid Analyzer, Model 120C (Moore, Speckman, and Stein, 1958). Protein samples were placed in 20 ml ampoules. Four mg of protein was used weighed within 0.1 mg. Eight ml of 6 N hydrochloric acid were added to the ampoules. The contents of the ampoules were frozen in a dry ice-ethanol bath. The ampoules were evacuated with a high vacuum pump, removed from the dry ice-ethanol bath and allowed to melt slowly to remove any gases in the frozen samples. The freezing and thawing procedure was repeated 2 times, after which they were sealed, while frozen, with an air-propane flame. The sealed ampoules were placed in an oil bath in a forced draft, recirculating oven regulated at 100 ± 2 C. The proteins were hydrolyzed for 20 and 70 hr. The ampoules were removed from the oven, cooled and stored in a deep freeze until used.

The hydrolysate was transferred quantitatively to a 25 ml pear-shaped flask fitted with a ground glass joint. The hydrolysate was evaporated to dryness on a rotary evaporator, redissolved in a small amount of deionized water and redried. The dried hydrolysate was dissolved in citrate HCl buffer (pH 2.2) and the final volume adjusted to 5 ml. An aliquot was removed for application to the amino acid analyzer. Standard amino acid mixtures were analyzed using the same ninhydrin solution within the same four-day period.

Amino Acids and Peptides--
Enzymatic Hydrolysates

The trichloroacetic acid supernatant from pepsin-pancreatin hydrolysates of protein preparations were evaluated for amino acid and peptide content after removal of trichloroacetic acid.

Amino acids and peptides were measured in the three-solvent single dimension thin-layer chromatography (TLC) system described by Pataki (1968). The solvent systems were those utilized by Wollenweber (1962) and by Bujard and Mauron (1966). The procedures and solvents for TLC of amino acids are summarized in Table 1. Solvents were redistilled, water was glass redistilled and the glacial acetic acid and ammonium hydroxide were fresh reagent grade. Separation was accomplished on a stationary phase of Machery and Nagel 300 cellulose powder (MN-300

cellulose). Glass plates of dimensions 20 x 20 cm were spread with a 0.25 mm thickness of MN-300 cellulose slurry. The slurry was prepared for five plates by homogenization of 15 g of MN-300 cellulose powder with 90 ml deionized water in a Waring Blender. After spreading, the plates were air dried overnight and activated for 10 min at 100 C in a dry air oven. The plates were developed in solvent tanks with a saturated atmosphere at room temperature until the solvent front had proceeded 18 cm.

Visualization was accomplished by spraying with 0.2% ninhydrin solution in n-butanol prepared fresh each day. The color was developed in an oven at 100 ± 2 C for 10 min. Quantization of the plates was accomplished by use of a recording densitometer with the technique described by Squibb (1963a, 1963b). The equipment used consisted of a Photovolt Thin-Layer plate scanner (Model 52C) attached to a Photovolt Recording Electrophoresis Densitometer (Model 542) with an Integraph Automatic Integrator (Model 49). Standard curves were prepared from thin-layer plates developed in the same fashion with incremental known amounts of amino acids to be measured. Quantitative relationships were established by relative areas as measured by integration. Peptides were noted, unique to each sample, and identified by Rf value for the solvent involved. Relative quantities were established by measurement of areas according to the lysine standard curve.

Measurement of Enzymatic Hydrolysis

Gel Filtration

Proteins were digested during gel filtration to remove the digestion products from the site of action of the digesting enzyme. The method proposed by Ford and Salter (1966) was used with modifications.

Chromatographic columns were prepared of 2.25 cm inside diameter and approximately 40 cm length. The columns were jacketed by wrapping with 1/4-inch tygon tubing in circuit with a circulating water bath. The columns were coated with dimethyldichlorosilane to insure a uniform flow rate at the glass-gel interface. Gel beads (Bio-Gel P-2) were hydrated in phosphate buffer (pH 7.6, 0.02 M) for 24 hr at 37 C. The columns were poured to a gel height of 21 cm to yield a void volume of 28.0 ml. Flow rate was adjusted by adjustment of head pressure with a 5 liter aspirator bottle fitted with a Mariotte constant pressure tube. The elution volumes for proteins and amino acids were established by adding known quantities of protein (β-lactoglobulin) and an amino acid (tyrosine). The effluent was monitored at 280 nm and the fraction volume established to include the desired component.

Continuous absorption at 280 nm resulted in a lack of resolution as hydrolysis allowed continuous release of amino acids. Volumetric fractions were established for a

protein without hydrolysis and for an amino acid. The volumetric fractions were based on elution of β-lactoglobulin in the 12-18 ml volume following the void volume and elution of tyrosine at 74-80 ml following the void volume. Fraction 1 represents the 28 ml quantity following the void volume to contain intact protein. The second 28 ml quantity following the void volume was designated as Fraction 2 and contained peptides and slowly hydrolyzed amino acids. Volume designated Fraction 3 was the last 50 ml volume collected and contained the compounds (amino acids and peptides) included in the gel, of molecular weight less than 2600. Temperature was maintained at 37 C ± 0.2 C in the jacketed columns.

Sample addition was performed by addition of protein solution containing 100 mg protein mixed immediately before application with 2 mg trypsin. Flow rate was adjusted with 0.02 M phosphate buffer, pH 7.6, to enable an elution time of 4 hr ± 15 min. Fractions were collected manually, monitored for both time and volume. Enzyme blanks were prepared in a manner analogous to the samples, including collection and measurement of volumetric fractions.

Pepsin and Pancreatin

The degree and form of protein hydrolysis was measured using the pepsin-Pancreatin Digest of Akeson and Stahmann (1964). Pepsin followed by pancreatin digests were prepared by incubating samples containing 100 mg

protein with 1.5 mg pepsin in 15 ml of 0.1 N hydrochloric acid at 27 C for 3 hr. The samples were adjusted to pH 8 with 0.2 N sodium hydroxide. After addition of 4 mg pancreatin in 7.5 ml phosphate buffer (pH 8, 0.1 M), the samples were incubated for an additional 24 hr at 37 C. Enzyme blanks were prepared under the same conditions with the protein sample omitted. Upon completion of the digestion, 25 ml of a 10% trichloroacetic acid (TCA) solution were added to the digest, and the final volume adjusted to 50 ml. Undigested protein was removed by centrifugation, and the supernatant was passed over an anion exchange resin (AG 2 x 8) in the chloride form to remove TCA. Samples were immediately dried in a rotary vacuum evaporator. The residue was dissolved in a small amount of citrate-HCl buffer (pH 2.2), and the volume was adjusted to 5 ml for measurement of amino acids and peptides.

The pepsin-pancreatin digestion was measured in duplicate preparations by measurement of TCA supernatant protein. One milliliter aliquots of the digest samples were added to tubes containing 1 ml of 10% trichloroacetic acid at incremental times. The supernatant protein content was determined after centrifugation using the method of McDonald (1965) described previously.

Proton Release by pH-Stat

The Sargent Recording pH-Stat was employed to measure proton release as the uptake of standardized

sodium hydroxide to maintain constant pH. The procedures described by Jacobsen et al. (1947) with conditions stipulated by Fasold and Gundlach (1963) were used.

The pH-stat was standardized at pH 8 with the Sorenson buffer described in Kolthoff and Laitenin (1948). At temperature 37 C, pH 8.00 was attained with a mixture of 5.75 ml of 0.05 M borax with 4.25 ml 0.10 N hydrochloric acid. Samples were prepared with a known protein content within the range 2-50 mg. The pH of samples was adjusted to 8.0 prior to storage at 4 C.

Enzyme solutions were prepared containing 0.083 mg trypsin or 0.240 mg pancreatin per ml. The enzyme preparations were stored at 4 C after adjustment of the pH to 8.0. Storage times of no longer than 2 hr were necessary to prevent enzyme autolysis.

Immediately prior to measurement of each preparation, an aliquot of the sample was placed in a 37 C water bath to increase the temperature to that for optimum enzyme activity. In a separate beaker 2-3 ml of enzyme solution were held under the same conditions. The substrate protein concentration was varied by quantitative dilution of the preparations of known protein content. The final volume applied to the pH-stat was 5 ml. Each 5 ml substrate sample was allowed to equilibrate to 37 C and pH 8 in the pH-stat during baseline settings. Electrode control was activated after equilibration, and 1 ml of pH adjusted

enzyme solution at 37 C was added. During the reaction in the pH-stat reaction vessel, 0.0502 N sodium hydroxide was added to maintain the pH at 8.00. The volume of sodium hydroxide required to maintain the desired pH was automatically recorded relative to time of the reaction.

Initial velocity for each reaction was estimated as the slope of the recorded curve. The line slope was assessed as that normal to the plane of a mirror when the curve was continuous in the mirror (Bergmeyer, 1963). Substrate concentrations were derived as molar quantities using the molecular weights for milk proteins compiled by McKenzie (1967). (Casein = 33,600, α_s-casein = 28,000, k-casein = 28,000, β-lactoglobulin = 18,000).

Reaction calculations were made using the double reciprocal plot technique essentially as treated by Lineweaver and Burk (1934) for pure enzyme-substrate systems. In these applications, the velocity and substrate terms represent emperical values since Dixon and Webb (1964) explain that only pure systems can be characterized in absolute terms.

TABLE 1.--Summary of the thin-layer chromatographic procedure for amino acids.

Amino Acid	Solvent	Rf
Lysine	Butanol/Glacial Acetic Acid/Water 4:1:5 (v/v) (Wollenweber, 1962)	0.42
Aspartic Acid		0.46
Serine		0.50
Glutamic Acid		0.35
Glycine		0.48
Alanine		0.38
Cystine		0.14
Valine		0.58
Isoleucine		0.67
Leucine		0.70
Tryptophan		0.62
Histidine	Methanol/Water/Pyridine 20:5:1 (v/v) (Wollenweber, 1962)	0.27
Arginine		0.04
Threonine		0.53
Proline	Chloroform/Methanol/17% Ammonia 2:2:1 (v/v) (Bujard and Mauron, 1966)	0.75
Methionine		0.81
Tyrosine		0.62
Phenylalanine		0.89

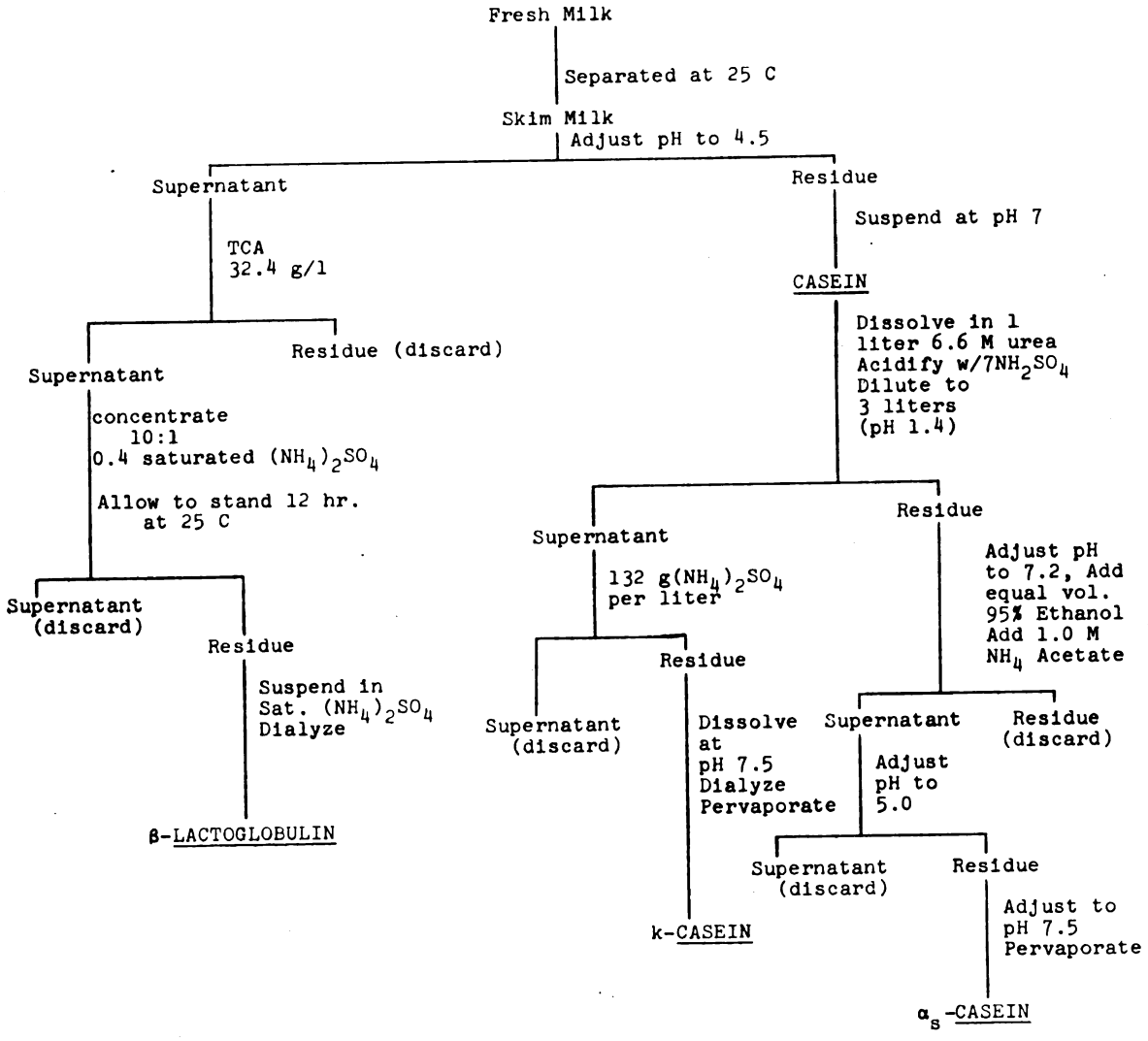

Figure 1.--Procedure for the preparation of milk protein fractions.

RESULTS

Milk Protein Preparations

Amino Acids

Amino acid analysis of the protein preparations was conducted after acid hydrolysis to verify the composition of the proteins studied. The amino acid compositions are compared to published amino acid contents of similar proteins in Table 2. Comparison of the amino acid contents to published results indicates that the preparations are essentially the same. The descrepancies noted can be associated with differences in preparative technique, analytical methods and inherent variation in milk proteins.

Characteristics of Protein Preparations upon Heat Treatment

The milk protein preparations each demonstrated a unique set of characteristics upon heat treatment. Changes included color development and pH differences after heat treatment, both alone and with lactose added. Before heat treatment each of the protein preparations was white. The pH was adjusted to 7 when necessary.

Casein demonstrated no observable color change associated with heat treatment alone, but when heated in the

presence of lactose, a uniform light tan color was observed. The pH of casein after heat treatment was 6.2 to 6.3 for both heated samples and those heated in the presence of lactose.

The color of α_s-casein was not perceptibly changed after heating; however heat treatment with added lactose resulted in a light tan color similar in intensity to casein. The pH of α_s-casein was 6.3 to 6.4 in samples heated alone and heated in the presence of lactose.

The color observed in heated k-casein preparations was a very faint tan. There was no discernable color difference between heated k-casein and that heated in the presence of lactose. The pH of k-casein after heat treatment was 5.7 to 5.8 in preparations heated alone and heated with added lactose.

The color observed in heated β-lactoglobulin preparations was more pronounced than in any of the heated casein samples. The color was gray-brown, compared to the tan in heated casein preparations. The pH of heated β-lactoglobulin preparations was 6.5 to 6.6 which represented less change from the original pH 7 than any other proteins observed.

β-lactoglobulin showed distinct coagulation after heating in the presence of added sodium chloride, added calcium and pH adjustment.

Pepsin-Pancreatin Digest of Proteins and Heat-Treated Proteins

Amino Acids and Peptides

The amino acids liberated by pepsin-pancreatin proteolysis of milk protein preparations are reported in Tables 3 to 6. Table 3 contains amino acid results for casein, 4 for α_s-casein, 5 for k-casein and 6 for β-lactoglobulin. The results for peptides estimated for the same milk protein preparations are presented in Table 7, designated by Rf values and expressed as lysine.

Amino acids and peptides in the unheated protein digests were assessed by analysis on an amino acid analyzer. The amino acid analyzer could not be used for the enzymatic hydrolysates of heated proteins and protein-lactose preparations. The ion-exchange resins evidently were unable to accept the digest residues other than amino acids (peptides and complexes) contained in hydrolysates of heated protein preparations. One digest of a heated sample could be satisfactorily eluted; however application of a second digest resulted in excessive volumn pressure buildup (200-300 lb). The residual material responsible for excessive pressure could not be removed by normal regeneration procedures. The contamination necessitated removal of resin from the column, washing and repacking. An ion-exchange resin designed for peptide analysis may have made use of the analyzer possible for digests of

heated preparations. An alternate method for amino acid assessment utilized quantitative thin-layer chromatography. The amino acid results for individual protein digests reported in Tables 3 to 6 represent analyses conducted with quantitative thin-layer chromatography. The amino acid results for control (unheated) protein preparations represent analyses conducted by thin-layer chromatography and essentially substantiated by the automatic amino acid analyzer (within ± 10 per cent).

The free amino acids from enzymatic digests of casein preparations are reported in Table 3 as quantities and as per cent of the total amino acid composition. The amino acid release by proteolysis was less from heated casein than from unheated casein and further reduced from casein heated in the presence of lactose for 10 of the 19 amino acids. Lysine, arginine and isoleucine were, on the contrary, released in greater amounts in enzymatic hydrolysates of casein heated in the presence of lactose than of unheated preparations.

An essential amino acid and peptide profile for the digest of casein preparations is Figure 2. The relative differences in the amounts of enzymatically hydrolyzed amino acids are pronounced, particularly decreases in threonine, valine, methionine and leucine released from casein heated in the presence of lactose. The six peptides measured were all present in greater amounts in

hydrolysates of heated than of unheated casein. The peptide designated 31 was present in the greatest relative amount for the digest of heated casein; while peptide 86 was predominant in the hydrolysate of casein heated in the presence of lactose.

The free amino acid contents of α_s-casein hydrolysates are reported in Table 4 as total amounts and as per cent of casein amino acid composition. The amino acids in α_s-casein hydrolysates of heated preparations were less than the unheated preparation for eight of the amino acids, and no significant change was noted for five amino acids.

Figure 3 represents the essential amino acids and peptides as profiles for enzymatic digests of α_s-casein preparations. The essential amino acid pattern formed for α_s-casein digests of heated and control preparations further indicates relatively smaller changes associated with heating in α_s-casein than in casein. The hydrolysate of α_s-casein heated in the presence of lactose contained smaller amounts than the control hydrolysate of histidine, threonine and leucine; however larger amounts were noted for valine and phenylalanine. The peptide contents of α_s-casein preparation digests were, as in the casein preparations, larger for heated than for unheated protein preparations.

The pepsin-pancreatin digest free amino acids from k-casein are reported in Table 5 as total amounts and as per cent of k-casein amino acid composition. The relative stability of k-casein to heat is indicated by the lack of significant difference in nine of the amino acids between heated and unheated preparation digests.

k-Casein essential amino acids and peptides from enzymatic digests for unheated and heated protein preparations are described in Figure 4. The relative amounts of histidine and threonine in digests of k-casein heated in the presence of lactose are notable as they represent slightly more than half the amounts in corresponding samples from control preparations. The peptide designated 31 was present in much greater quantity in the digest of heated k-casein than in digests of the unehated protein or k-casein heated in the presence of lactose. Peptide 68 was present in increased quantity in the digest of k-casein heated in the presence of lactose relative to the unheated preparations.

The free amino acids and peptides from digests of β-lactoglobulin preparations are in Table 6, presented as total quantities and as percentages of amino acid composition. The amino acids in enzymatic digests from heated β-lactoglobulin preparations were present in greater amounts in eight cases and lesser amounts in six amino acids than in the control (unheated) preparations.

The essential amino acids and peptides from digests of β-lactoglobulin (Figure 5) illustrate inconsistent amino acid release upon proteolysis associated with heat treatment. The amino acids valine and histidine were released in considerably smaller amounts in the heated preparation digests than in unheated counterparts. The peptides from pepsin-pancreatin digests of β-lactoglobulin were unique among the proteins examined as peptides were released in greater quantities from heated protein than from protein heated in the presence of lactose, except peptide 86.

Trichloroacetic Acid-Soluble Proteolysis Products

The enzymatic cleavage of milk protein preparations during pepsin-pancreatin proteolysis was monitored by assessment of the trichloroacetic acid-soluble hydrolysis products. The soluble proteins released by stepwise hydrolysis during first pepsin, then pancreatin proteolysis are reported for milk protein preparations in Table 8. The graphic descriptions of enzymatic proteolysis products from individual milk proteins for unheated and heated preparations are in Figures 6 through 9. The order of appearance of milk protein preparations in both tabular and graphic presentation is: casein, α_s-casein, k-casein and β-lactoglobulin. All measurements

were made on the same sample for the individual protein preparations. The transition from pepsin to pancreatin represents adjustment of the pH from 2 to 8 in the protein solution and the addition of pancreatin.

The changes in appearance of the protein preparations during hydrolysis were conspicuous and consistent. Initially all preparations were turbid and individually colored as previously described. Within 1 hr after initiation of proteolysis turbidity had disappeared from all samples. The heated samples which were tan-colored upon initiation of proteolysis became a clear brown. The unheated α_s-casein preparation had a distinct blue cast which was not observed in the heated α_s-casein sample or in other unheated protein preparations.

Enzymatic Proteolysis During Gel Filtration

Total Protein Distribution

Milk protein preparations; unheated, heated and heated in the presence of lactose were fractionated during tryptic proteolysis on an acrylamide desalting gel (Bio-Gel P-2). The protein contents of the fractions derived are reported in Table 9; including Fraction 1--intact protein, Fraction 2--large peptides and slowly hydrolyzed amino acids and Fraction 3--small (MW less than 2600) peptides and amino acids.

The protein quantities contained in the intact protein portion of the enzymatic hydrolysates were 50-100% greater from heated casein and 22-41% greater from heated α_s-casein than from the corresponding unheated protein preparations. The protein contents of the intact protein fractions were less than 10% different from heated and unheated k-casein preparations. The intact protein after tryptic proteolysis of β-lactoglobulin was less than half in heated preparations of that in the unheated protein. The amino acid and peptide-containing fractions (Fractions 2 and 3) were complementary to the intact protein, as the sums of the protein contents were similar for all protein preparations examined.

Trichloroacetic Acid-Soluble Nitrogenous Material

The extent of enzymatic proteolysis for each of the model proteins during gel filtration hydrolysis was estimated by measurement of the Lowry-Folin reacting material in trichloroacetic acid supernatants of intact protein, peptide and amino acid fractions. These data are reported in Table 10. The trichloroacetic acid-soluble protein contents of Fraction 1 (intact protein) are considerably greater in heated preparations of casein (15 go 20 times) and of α_s-casein (3 to 4 times) than the corresponding unheated (control) proteins. The trichloroacetic acid supernatant protein of the k-casein

intact protein fraction was influenced much less by heat treatment, with a 25% increase noted only after heat treatment in the presence of lactose. The trichloroacetic acid-soluble protein from the intact protein fraction after gel-filtration proteolysis of β-lactoglobulin was half the amount from heated protein as it was from the unheated control. There was, therefore, a correlation between trichloroacetic acid-soluble protein and total protein which was also prevalent in Fraction 2 and Fraction 3.

Reaction with 1-Fluoro-2, 4-dinitrobenzene

The quantities of materials reactive with 1-fluoro-2, 4-dinitrobenzene (FDNB) are reported in Table 11 for protein preparations before gel-filtration proteolysis and for the fractions obtained after gel-filtration hydrolysis. The unit for expression of FDNB reactive materials in proteins and hydrolysis fractions was adapted to terminal amino rather than the usual ε-amino connotation, even though εNH_2-DNP-lysine was used as the basic standard. The expression terminal amino groups more precisely describes the reaction measured than ε-amino lysine. There was up to 36 times as much FDNB reacting material in gel-filtration hydrolyzed proteins as in the same protein preparations before hydrolysis.

The terminal amino groups measured in milk protein preparations before hydrolysis were essentially the same, irrespective of heat treatment, with the exception of heat-treated β-lactoglobulin which was 17% greater than the unheated protein. The terminal amino groups of the intact protein fraction (Fraction 1) derived after gel-filtration hydrolysis of proteins varied in the same way as the total protein contents of corresponding protein preparations. The terminal amino groups for peptides and amino acids (Fractions 2 and 3) from gel filtration proteolysis reflected the amino groups reactive with FDNB, and were erratic but in all cases amounted to more than the lysine content. The amounts of FDNB reacting material in excess of the lysine content indicated the reaction with α- and ε-amino groups.

Rate of Proteolysis by pH-Stat

Evaluation of protein preparations during proteolysis by monitoring proton release provides a means for examination of the substrate differences by the kinetics of hydrolysis. A more extensive examination of protein treatments influencing proteolysis was possible with use of the pH-stat than by the more involved techniques previously reported.

The expression terms for results of proteolysis-rate measurements are substrate concentration and specific activity. The more familiar counterparts for homogeneous

systems are K_m for substrate concentration and V for specific activity.

Equal Cleavage Sites

The influence of trypsin cleavage site concentration was evaluated at equal lysine and arginine concentration for each model protein by enzymatic hydrolysis rate measurement. Expressions of results for rate measurements in Table 12 are in terms of number of trypsin cleavage sites rather than the conventional molar concentration.

The differences between rate measurements for model protein preparations reflect individual protein behavior other than that attributable to the number of cleavage sites. The specific activity, or reaction velocity, for β-lactoglobulin control (unheated) was less than one-third that for α_s-casein control and half that for casein and k-casein controls. The unheated proteins also demonstrated individual cleavage rate characteristics independent of the number of cleavage sites, with a range of specific activity from 1.0 to 26.6. Protein preparations heated in the presence of lactose reflected behavior similar to heated preparations with a range in specific activity from 7.0 to 30.9. The lack of similarity of rate measurements for individual protein preparations indicated that numbers of cleavage sites did not govern differences in rates of proteolysis.

Chemical Denaturing Agents

The influences of chemical denaturing agents; urea, 2-mercaptoethanol and performic acid on rates of proteolysis are reported in Table 13 to 16. Reaction rate measurements of casein are in Table 13, α_s-casein in Table 14, k-casein in Table 15 and β-lactoglobulin in Table 16. The rates of proteolysis after heat treatment at pH 5 and 9 are also reported for each protein. Enzymatic proteolysis rate for each protein fraction subjected to a chemical denaturing agent was assessed unheated, after heat treatment and after heat treatment in the presence of lactose. Urea treatment was evaluated for only casein and β-lactoglobulin, as α_s-casein and k-casein were treated with urea as a part of their fractionation.

Casein demonstrated no difference in specific activity between urea-treated and untreated preparations. The specific activity for urea-treated casein was twice as great, however, after heat treatment and was five times as great after heat treatment in the presence of lactose.

β-lactoglobulin treated with urea demonstrated an activity twice that observed in the untreated preparation. The urea-treated β-lactoglobulin also reflected more rapid hydrolysis after heat treatment, double the specific activity for the corresponding untreated protein. A slight reduction in hydrolysis rate was noted for

urea-treated β-lactoglobulin after heat treatment in the presence of lactose.

Casein treated with 2-mercaptoethanol demonstrated a reduced velocity of enzymatic proteolysis, one-tenth the specific activity of the untreated casein preparation. Casein treated with 2-mercaptoethanol and then heated demonstrated a five-fold increase in activity and when heated in the presence of lactose resulted in an increase in specific activity of 25%.

β-Lactoglobulin treated with 2-mercaptoethanol was enzymatically hydrolyzed less rapidly than the untreated protein with a specific activity one-third as great for treated as untreated proteins. The specific activity for 2-mercaptoethanol-treated, then heated β-lactoglobulin was two-thirds that of the untreated, but an increase of 10% was observed in the specific activity for the 2-mercaptoethanol-treated protein heated in the presence of lactose from the corresponding untreated preparation.

Performic acid treatment of casein resulted in increased rates of proteolysis, measured as specific activity for all protein preparations; unheated, heated and heated in the presence of lactose.

α_s-Casein treated with performic acid was hydrolyzed 25% more rapidly than the untreated protein, indicated by specific activity. The proteolysis velocity of performic acid-treated α_s-casein after heat treatment was reduced to

one-third the control protein, but heat treatment in the presence of lactose resulted in a slight increase in the rate of enzymatic hydrolysis.

k-Casein proteolysis velocity was reduced in all cases by performic acid treatment for unheated and heat-treated preparations.

Performic acid-treated β-lactoglobulin was hydrolyzed more rapidly by approximately 30% in all cases, indicated by specific activity assessment compared to control protein preparations.

Heat treatment at pH 5 resulted in reduction of enzymatic proteolysis to immeasurable quantities for casein and α_s-casein. k-Casein and β-lactoglobulin were hydrolyzed more rapidly after heat treatment at pH 5 as evidenced by a 50% increase in specific activity for k-casein and a slight increase for β-lactoglobulin, compared to corresponding untreated proteins.

Heat treatment at pH 9 resulted in decreases in rate of proteolysis for each of the proteins compared to their untreated controls.

The treatment of protein preparations with 0.3 M calcium is reported as a treatment for individual proteins in Tables 13 through 16. The presence of calcium ions in casein and β-lactoglobulin preparations resulted in greater enzymatic proteolysis velocity in all cases. The rate of proteolysis indicated by specific activity for

calcium-treated α_s-casein preparations was less than half the velocity for preparations not containing calcium. k-Casein preparations containing calcium were hydrolyzed less rapidly in each preparation, but the reduction was not pronounced.

Protein treatments with 0.15 M sodium chloride (isotonic saline) as they influenced rates of proteolysis are reported as one of the treatments for each protein in Tables 13 through 16. Sodium chloride treatment apparently stabilized the protein preparations to heat treatment, but the specific activity for isotonic saline-treated protein preparations was less than the specific activity for the untreated control proteins.

Sugars

The sugars; glucose, lactose, galactose and glucose plus galactose are reported as treatments to evaluate their influence on enzymatic proteolysis rate of casein in Table 13 and of β-lactoglobulin in Table 16. The enzymatic hydrolysis rate for casein was greater for each sugar-treated casein preparation than it was for untreated casein. The specific activity for sugar-casein preparations was less after heat treatment than before, but only after heat treatment in the presence of lactose was the rate less than heated untreated casein.

β-lactoglobulin solutions containing added sugars reflected rates of proteolysis 5-20% higher than untreated

protein. β-lactoglobulin heat treatment in the presence of sugars, however, resulted in specific activity measurements less than or equal to velocities observed with no added sugar.

Control Measurements

Control measurements (blanks) were made for each of the variables, and in several cases, the observations are pertinent to interpretation of results.

Each of the protein preparations; casein, α_s-casein, k-casein and β-lactoglobulin was examined for proteolytic enzymes inherent to the proteins by monitoring proton release without added enzyme. Proteolysis was not measureable by pH-stat in any protein preparation examined.

Lactose concentration was evaluated within the range of solubility for possible changes in enzymatic proteolysis rate. Within the range of lactose concentration (0.06 M to 0.24 M) there was no observed difference in enzymatic proteolysis rate monitored by proton release.

Enzyme autolysis was considered in each experiment, and corrections were made when indicated. Amino acid analyses were corrected to compensate for the enzyme contribution of each amino acid established by separate enzyme blank analysis. The activity of prepared enzyme solutions was evaluated during storage and autolysis by the enzymes made it necessary to prepare fresh solutions after no more than 2 hr storage at 4 C.

TABLE 2.--A comparison of the amino acid compositions of the milk proteins utilized in this study with those reported by others.[a]

Amino Acid Residue	Casein		α_s-Casein		k-Casein		β-Lacto-globulin	
	This Study	(1)	This Study	(2)	This Study	(3)	This Study	(4)
				(%)[b]				
Lys	8.9	7.1	8.3	8.2	5.8	5.6	28.1	28.5
His	3.4	2.6	1.8	2.9	2.1	2.0	1.1	1.4
Arg	4.1	3.7	4.0	3.9	3.6	3.5	2.1	2.6
Asp	7.0	5.6	6.3	6.8	6.2	6.6	9.8	9.7
Thr	3.9	3.8	2.5	2.5	5.7	5.7	4.5	4.4
Ser	4.6	5.2	4.8	5.3	4.2	4.1	3.1	3.1
Glu	23.0	20.2	22.8	21.4	19.4	17.3	17.0	17.1
Pro	10.6	10.3	8.4	6.9	8.9	9.1	4.4	4.5
Gly	1.6	1.6	2.1	2.1	0.8	0.9	1.2	0.9
Ala	2.8	2.4	2.8	2.6	4.5	4.3	5.4	5.3
1/2 Cys	0.0	0.3	0.1	0.0	0.8	0.9	1.8	1.8
Val	6.3	6.3	5.2	4.7	5.2	5.3	5.0	5.3
Met	2.0	2.9	1.8	2.2	1.1	1.1	1.9	2.2
Ile	5.4	5.7	5.5	5.5	5.9	6.0	5.6	5.9
Leu	9.0	8.7	7.0	6.9	5.1	5.2	11.7	12.8
Tyr	5.6	5.7	7.0	7.0	6.2	6.8	3.4	3.5
Phe	5.2	5.2	5.2	3.9	3.3	3.4	3.4	3.2
Trp		2.5		7.3		5.5		2.0
Phosphorus				1.01		0.22		
Carbohydrate						1.4		
TOTAL N		15.4		15.3		15.3		8.8

[a]Analytical data compared with data reported in the following sources: (1) Block and Weiss (1956); (2) Gordon et al. (1965); (3) Swaisgood et al. (1964); (4) Kalan et al. (1965).

[b]Amino acids expressed as anhydrous residues.

TABLE 3.--Free amino acids in pepsin-pancreatin digest of casein and heat-treated casein.

Amino Acids		Treatment					
Residue	Composition[a] of Casein (μ moles/g)	Control (μ/moles/g)	(%)[c]	Heated[b] (μ/moles/g)	(%)[c]	Heated in Presence of Lactose[b] (μ moles/g)	(%)[c]
Essential							
Lys	85.4	39.0	46	33.2	39	50.8	59
His	31.1	4.4	14	3.9	13	1.9	6
Thr	47.3	30.6	64	39.0	82	19.5	41
Val	82.4	34.1	41	38.5	46	19.5	24
Met	19.8	20.9	105	20.7	104	8.4	42
Ile	59.1	19.5	33	27.3	46	50.7	86
Leu	97.8	48.8	49	19.8	20	20.1	20
Tyr	41.9	21.1	50	8.4	20	8.4	20

Phe	44.2	13.6	31	29.7	67	19.5	44
Trp	--	1.4	--	1.4	--	1.2	--
Non-Essential							
Arg	32.7	17.8	54	19.5	59	29.3	90
Asp	74.9	72.9	97	70.3	94	35.1	46
Ser	6.5	4.6	71	3.9	60	4.0	61
Glu	217.1	71.6	33	35.1	16	38.1	18
Pro	132.9	137.0	103	79.6	59	126.7	95
Gly	33.2	33.5	101	14.5	44	7.8	24
Ala	47.8	35.1	74	27.3	58	13.7	27
1/2 Cys	--	--	--	--	--	--	--

[a]Hydrolyzed in 6N HCl for 20 hr, analyzed with an amino acid analyzer.

[b]Heated at 120 C for 30 min.

[c]Expressed as percentage of total composition.

TABLE 4.--Free amino acids in pepsin-pancreatin digest of α_s-casein and heat-treated α_s-casein.

Amino Acids		Treatment					
Residue	Composition[a] of α_s-Casein (μ moles/g)	Control (μ/moles/g)	(%)[c]	Heated[b] (μ/moles/g)	(%)[c]	Heated in Presence of Lactose[b] (μ moles/g)	(%)[c]
Essential							
Lys	66.2	32.6	50	33.3	50	29.5	45
His	12.9	7.2	56	3.4	26	2.2	17
Thr	25.1	19.8	80	18.7	75	10.9	43
Val	52.3	50.6	97	50.2	96	50.7	97
Met	6.8	3.9	57	2.5	37	4.2	62
Ile	48.8	42.1	86	42.0	86	44.2	90
Leu	62.7	51.3	82	65.5	104	39.3	63
Tyr	43.2	35.9	83	27.3	63	30.7	71

Phe	36.3	23.1	64	24.1	66	28.9	80
Trp	--	1.7	--	1.7	--	1.7	--
Non-Essential							
Arg	25.9	24.6	95	25.6	98	17.1	66
Asp	54.4	53.3	97	54.0	99	42.5	78
Ser	54.4	34.1	63	35.6	65	35.6	65
Glu	176.6	77.5	44	77.5	44	77.5	44
Pro	88.2	12.4	14	9.3	11	9.4	11
Gly	37.5	17.8	48	12.4	33	12.6	34
Ala	40.3	37.1	92	31.9	79	29.5	73
1/2 Cys	--	--	--	--	--	--	--

[a]Hydrolyzed in 6H HCl for 20 hr, analyzed with an amino acid analyzer.

[b]Heated at 120 C for 30 min.

[c]Expressed as percentage of total composition.

TABLE 5.--Free amino acids in pepsin-pancreatin digest of k-casein and heat-treated k-casein.

Amino Acids		Treatment					
Residue	Composition[a] of k-Casein (μ moles/g)	Control (μ moles/g)	(%)[c]	Heated[b] (μ moles/g)	(%)[c]	Heated in Presence of Lactose[b] (μ moles/g)	(%)[c]
Essential							
Lys	90.2	40.5	45	38.2	42	45.0	50
His	28.1	24.7	88	23.0	82	14.4	51
Thr	97.7	52.9	54	45.0	46	29.2	29
Val	91.5	45.3	49	46.2	51	45.9	50
Met	19.9	12.5	63	12.2	17	10.4	52
Ile	93.0	15.7	17	15.7	17	13.2	14
Leu	80.4	78.4	97	72.0	89	72.0	89
Tyr	67.6	36.3	54	36.0	53	36.0	53

Phe	39.3	38.7	98	40.5	103	38.3	97
Trp	--	1.7	--	1.9	--	2.3	--
Non-Essential							
Arg	42.1	22.5	53	33.7	80	22.5	53
Asp	101.6	92.3	91	94.7	93	87.9	86
Ser	86.6	36.8	42	36.3	42	36.7	42
Glu	266.6	153.1	57	157.0	59	157.0	59
Pro	162.5	147.4	91	97.1	60	104.7	64
Gly	25.6	24.4	95	23.0	89	23.1	90
Ala	111.8	85.0	76	82.2	73	82.6	73
1/2 Cys	13.8	12.0	87	12.1	88	12.1	88

[a]Hydrolyzed in 6N HCl for 20 hr, analyzed with an amino acid analyzer.

[b]Heated at 120 C for 30 min.

[c]Expressed as percentage of total composition.

TABLE 6.--Free amino acids in pepsin-pancreatin digest of β-lactoglobulin and heat-treated β-lactoglobulin.

Amino Acids		Treatment					
Residue	Composition of[a] -Lactoglobulin (μ moles/g)	Control (μ moles/g)	(%)[c]	Heated (μ moles/g)	(%)[c]	Heated in Presence of Lactose[b] (μ moles/g)	(%)[c]
Essential							
Lys	356.1	274.0	77	333.0	93	294.0	82
His	118.5	98.2	83	74.2	63	70.2	59
Thr	74.5	26.5	35	49.0	66	49.0	66
Val	81.9	87.2	106	56.8	72	46.1	56
Met	26.0	23.4	90	23.1	89	21.2	81
Ile	78.7	68.2	87	74.9	95	69.3	88
Leu	167.0	129.3	77	137.2	82	129.4	77
Try	33.7	34.6	102	35.1	104	38.4	113

Phe	39.0	34.6	89	34.8	89	39.0	100
Trp	--	0.6	--	0.9	--	1.2	--
Non-Essential							
Arg	12.5	10.7	86	12.0	96	11.5	92
Asp	139.2	135.0	97	136.8	98	125.6	90
Ser	59.8	41.3	69	41.1	69	45.1	75
Glu	212.8	117.0	55	101.2	48	137.2	64
Pro	76.4	14.5	19	20.5	27	22.5	29
Gly	36.6	30.2	82	22.1	60	34.6	94
Ala	122.2	107.5	88	104.7	86	114.7	93
1/2 Cys	39.1	34.6	88	30.8	79	17.3	44

[a]Hydrolyzed in 6N HCl for 20 hr, analyzed with an amino acid analyzer.

[b]Heated at 120 C for 30 min.

[c]Expressed as percentage of total composition.

TABLE 7.--Peptides in pepsin-pancreatin digest of milk proteins and heat-treated milk proteins.

Peptide	Casein			α_s-Casein			k-Casein			ß-Lactoglobulin		
	Control	Heated[a]	Heated in[a] Presence of Lactose	Control	Heated[a]	Heated in[a] Presence of Lactose	Control	Heated[a]	Heated in[a] Presence of Lactose	Control	Heated[a]	Heated in[a] Presence of Lactose
	(u mole Lys/g)[b]											
R_f 31[c]	0.0	37.5	12.5	0.0	10.0	13.7	0.0	97.5	12.5	0.0	62.5	0.0
R_f 68[c]	0.0	16.2	16.2	0.0	18.7	32.5	0.0	17.5	23.7	0.0	51.0	25.0
R_f 42[d]	12.5	23.7	18.7	10.0	18.4	16.2	5.0	18.7	18.5	15.0	17.5	16.5
R_f 95[d]	5.0	15.0	7.5	1.2	16.2	16.5	12.5	13.7	0.0	15.0	14.0	5.0
R_f 74[e]	5.0	18.7	16.2	1.2	1.7	3.2	16.2	16.3	17.5	13.7	33.7	23.7
R_f 86[e]	13.7	16.2	32.5	11.2	18.7	26.2	16.2	15.0	18.7	15.0	32.5	31.2

[a]Heated at 120 C for 30 min.

[b]Relative quantities expressed to lysine standard.

[c]Designated by R_f in methanol/water/pyridine TLC solvent system.

[d]Designated by R_f in chloroform/methanol/ammonia TLC solvent system.

[e]Designated by R_f in butanol/acetic acid/water TLC solvent system.

TABLE 8.--Trichloroacetic acid soluble protein measured at intervals during pepsin-pancreatin digest of milk proteins and heat-treated milk proteins.

Enzyme / Time	Casein			α_s-Casein			k-Casein			β-Lactoglobulin		
	Control	Heated[b]	Heated in[b] Presence of Lactose	Control	Heated[b]	Heated in[b] Presence of Lactose	Control	Heated[b]	Heated in[b] Presence of Lactose	Control	Heated[b]	Heated in[b] Presence of Lactose
	(mg TCA sol/100 mg)[c]											
Pepsin												
Initial	1.1	7.2	6.5	4.7	8.8	11.6	2.9	15.3	18.7	1.9	10.7	5.3
10 min	6.3	15.3	12.9	8.4	12.6	25.2	13.1	16.1	15.1	23.4	6.4	6.8
20 min	11.7	22.3	16.5	9.8	14.0	31.7	21.4	21.5	19.9	27.6	6.6	8.3
30 min	13.6	22.4	23.4	11.6	18.6	31.7	21.5	21.8	20.7	27.7	7.0	12.1
1 hr	17.1	22.1	23.4	15.3	31.2	31.7	23.6	23.1	19.7	27.7	14.2	12.9
3 hr	23.0	24.0	24.1	29.8	31.2	31.7	23.9	24.7	22.2	27.7	14.7	14.8
Pancreatin												
1 hr[d]	45.9	50.0	36.4	51.8	50.4	42.2	56.4	54.2	33.8	39.1	20.9	29.7
2 hr	67.7	70.9	45.9	60.9	56.2	49.4	70.0	60.9	39.2	48.2	26.3	38.8
3 hr	85.1	81.2	62.1	70.4	61.2	51.7	78.8	66.6	41.8	60.1	31.4	46.1
8 hr	92.4	94.2	71.7	84.7	69.4	65.4	91.1	74.3	52.1	84.9	51.7	61.9
16 hr	95.4	94.2	75.3	88.4	78.1	77.4	94.7	80.4	64.8	91.5	70.4	80.4
24 hr	96.2	95.1	78.2	93.7	83.6	87.0	96.5	81.2	76.0	93.5	75.5	85.2
Recovery[e]	77.2	66.9	64.7	87.8	78.6	83.5	81.8	80.9	75.6	78.4	63.6	59.6

[a]Exposure period to designated enzyme, same samples used throughout.

[b]Heated at 120 C for 30 min.

[c]Expressed as mg trichloroacetic soluble nitrogen containing material per 100 mg protein.

[d]pH adjusted to 8 after pepsin digest.

[e]Quantity remaining after desalting gel.

TABLE 9.--Measurement of protein content in fractions of proteins and heat-treated proteins subjected to tryptic proteolysis during gel filtration.

Specimen	Casein			α_s-Casein			k-Casein			β-Lactoglobulin		
	Control	Heated[a]	Heated in[a] Presence of Lactose	Control	Heated[a]	Heated in[a] Presence of Lactose	Control	Heated[a]	Heated in[a] Presence of Lactose	Control	Heated[a]	Heated in[a] Presence of Lactose
	(mg/g)[b]											
Total Recovered	901	908	803	950	943	935	936	922	937	951	897	937
Fraction 1[c]	291	604	455	513	725	616	584	602	560	690	277	334
Fraction 2[d]	575	221	328	265	85	178	326	153	183	213	190	302
Fraction 3[e]	34	82	97	170	132	139	26	167	195	48	428	302

[a]Heated at 120 C for 30 min.

[b]Expressed as mg protein recovered per g protein applied.

[c]Intact protein excluded by gel.

[d]Peptide and slowly hydrolyzed amino acids.

[e]Amino acids and peptides included in gel.

TABLE 10.--Trichloroacetic acid soluble nitrogen containing material from proteins and heat-treated proteins subjected to tryptic proteolysis during gel filtration.

Specimen	Casein			α_s-Casein			k-Casein			β-Lactoglobulin		
	Control	Heated[a]	Heated in[a] Presence of Lactose	Control	Heated[a]	Heated in[a] Presence of Lactose	Control	Heated[a]	Heated in[a] Presence of Lactose	Control	Heated[a]	Heated in[a] Presence of Lactose
	(mg TCA sol/100 mg)[b]											
Initial	0.9	7.1	6.4	4.7	8.7	11.5	2.7	15.1	17.6	1.8	10.5	5.2
Fraction 1[c]	2.2	40.7	36.8	16.8	66.1	53.9	38.3	36.8	47.0	46.0	23.1	22.7
Fraction 2[d]	47.0	20.3	22.9	20.4	3.8	13.7	29.7	11.5	17.0	17.0	16.9	26.4
Fraction 3[e]	0.6	6.6	5.6	14.9	6.3	12.0	2.0	12.2	18.7	9.0	19.1	26.7

[a]Heated at 120 C for 30 min.

[b]Expressed as mg TCA soluble protein per 100 mg initial protein sample.

[c]Intact protein excluded by gel.

[d]Peptides and slowly hydrolyzed amino acids.

[e]Amino acids and peptides included in gel.

TABLE 11.--Terminal amino groups of proteins and heat-treated proteins subjected to tryptic proteolysis during gel filtration.

Specimen	Casein			α_s-Casein			k-Casein			ß-Lactoglobulin		
	Control	Heated[a]	Heated[a] in Presence of Lactose	Control	Heated[a]	Heated[a] in Presence of Lactose	Control	Heated[a]	Heated[a] in Presence of Lactose	Control	Heated[a]	Heated[a] in Presence of Lactose
	(mg ϵ-NH_2 DNP Lys/100g)											
Initial	148	143	141	133	129	133	145	143	142	138	165	142
Fraction 1[b]	555	275	278	403	179	96	214	208	183	156	746	360
Fraction 2[c]	252	172	261	74	418	70	542	256	198	278	311	236
Fraction 3[d]	3,090	1,190	80	80	327	49	528	219	423	197	230	254

[a]Heated at 120 C for 30 min.

[b]Intact protein excluded by gel.

[c]Peptide and slowly hydrolyzed amino acids.

[d]Amino acids and peptides included in gel.

TABLE 12.--Rates of proteolysis of protein fractions at equal cleavage site concentrations measured by proton release.

Treatment	Casein		α_s-Casein		k-Casein		β-Lactoglobulin	
	Substrate[a] Concentration	Specific[b] Activity	Substrate[a] Concentration	Specific[b] Activity	Substrate[a] Concentration	Specific[b] Activity	Substrate[a] Concentration	Specific[b] Activity
Control	6.2	22.4	8.7	35.6	4.2	26.1	23.0	11.2
Heated[c]	1.7	17.5	1.0	1.0	9.1	26.6	0.5	10.3
Heated in Presence of Lactose[c]	6.6	17.5	0.6	7.0	0.9	30.9	0.2	9.8

[a]Substrate concentration: $x10^{-5}$ available sites.

[b]Specific activity: $x10^{-5}$ sites/min.

[c]Heated at 120 C for 30 min.

TABLE 13.--Rates of tryptic proteolysis of casein and heat-treated casein after treatment with sugars, chemical denaturing agents and pepsin digest.

Treatment	Substrate Concentration			Specific Activity		
	Control	Heated[a]	Heated in[a] Presence of Lactose	Control	Heated[a]	Heated in[a] Presence of Lactose
	. . . (10^{-3} M)			. . . . (10^{-3} mM/min) . .		
Control	0.77	0.22	0.83	2.8	2.2	1.6
Heated pH 5		(f)			(f)	
Heated pH 9		0.20			1.9	
Glucose[b]	3.12	0.42		8.0	2.9	
Lactose[b]	1.35	0.83		5.0	1.6	
Galactose	0.80	0.83		4.0	3.4	
Glucose and Galactose[b]	2.50	0.46		6.2	2.9	
Urea[c]	0.58	0.43	1.11	2.9	4.9	10.0
Mercaptoethanol	0.53	3.22	0.25	0.2	10.0	2.5
Performic Acid	2.27	0.39	0.85	5.3	2.9	3.1
Calcium ions[d]	6.66	0.42	1.66	20.0	5.0	12.5
Scdium chloride[e]	0.33	0.33	0.33	2.5	3.3	2.5
Pepsin digest:	1.96	3.84	1.96	4.3	8.3	3.7
Heated pH 5		0.001			3.6	
Heated pH 9		(f)			(f)	

[a]Heated at 120 C for 30 min.
[b]Sugar concentrations - 0.06 M.
[c]Urea - 8 M.
[d]Calcium - 0.3 M.
[e]Isotonic saline - 0.15 M sodium chloride.
[f]No measurable proteolysis.

TABLE 14.--Rates of tryptic proteolysis of α_s-casein and heat-treated α_s-casein after treatment with chemical denaturing agents and pepsin digest.

Treatment	Substrate Concentration			Specific Activity		
	Control	Heated[a]	Heated in[a] Presence of Lactose	Control	Heated[a]	Heated in[a] Presence of Lactose
	. . . (10^{-3} M) . . .			. . . (10^{-3} mM/min) . .		
Control	1.88	0.78	0.15	7.7	7.7	1.5
Heated pH 5		(b)				
Heated pH 9		0.25			1.5	
Mercaptoethanol	1.80	0.78	0.15	7.3	7.8	1.5
Performic Acid	2.70	4.67	4.67	10.0	1.9	1.3
Calcium Ions[c]	1.92	2.22	0.39	4.2	4.2	1.1
Sodium Chloride[d]	1.00	0.41	0.50	2.0	1.6	1.6
Pepsin Digest:	0.30	0.21	0.20	1.1	1.1	1.7
Heated pH 5		0.30			0.6	
Heated pH 9		0.01			0.5	

[a]Heated at 120 C for 30 min.

[b]Reaction rate not measurable; no response to substrate concentration change.

[c]Calcium - 0.3 M.

[d]Isotonic saline - 0.15 M Sodium Chloride.

TABLE 15.--Rates of tryptic proteolysis of k-casein and heat-treated k-casein after treatment with chemical denaturing agents and pepsin digest.

Treatment	Substrate Concentration			Specific Activity		
	Control	Heated[a]	Heated in[a] Presence of Lactose	Control	Heated[a]	Heated in[a] Presence of Lactose
	. . . . (10^{-3} M) . . .			. . . . (10^{-3} mM/min) . .		
Control	0.57	0.78	0.57	3.6	3.6	7.7
Heated pH 5		12.50			5.0	
Heated pH 9		0.05			2.2	
Mercaptoethanol	0.25	0.41	0.50	1.3	2.0	10.0
Performic Acid	0.26	0.26	0.26	0.8	3.5	1.1
Calcium ions[b]	0.55	0.55	0.55	2.6	3.6	3.0
Sodium Chloride[c]	0.95	0.95	1.66	3.2	3.2	3.0
Pepsin Digest:	1.66	1.51	3.70	4.2	3.3	6.7
Heated pH 5		3.03			0.8	
Heated pH 9		3.84			4.8	

[a]Heated at 120 C for 30 min.

[b]Calcium - 0.3 M.

[c]Isotonic saline - 0.15 M sodium chloride.

TABLE 16.--Rates of tryptic proteolysis of β-lactoglobulin and heat-treated β-lactoglobulin after treatment with sugars, chemical denaturing agents and pepsin digest.

Treatment	Substrate Concentration			Specific Activity		
	Control	Heated[a]	Heated in[a] Presence of Lactose	Control	Heated[a]	Heated in[a] Presence of Lactose
	. . . (10^{-3} M)			. . . . (10^{-3} mM/min) . .		
Control	4.08	1.11	2.50	2.0	2.0	2.0
Heated pH 5		2.86			2.5	
Heated pH 9		1.33			1.8	
Glucose[b]	0.98	2.17		2.4	0.8	
Lactose[b]	1.25	2.50		2.4	2.0	
Galactose[b]	0.80	2.17		2.1	1.0	
Glucose and Galactose[b]	0.95	0.68		2.4	2.0	
Urea[c]	0.43	0.18	0.06	4.0	5.0	1.4
Mercaptoethanol	0.25	0.14	0.14	0.7	1.3	2.2
Performic Acid	0.96	0.27	0.51	3.3	3.3	2.9
Calcium ions[d]	1.20	2.00[f]	3.33[f]	3.3	6.2[f]	10.0[f]
Sodium Chloride	0.65	0.79[f]	2.50[f]	1.7	1.7[f]	1.7[f]
Pepsin digest:	0.95	2.38	4.54	3.3	3.3	5.8
Heated pH 5		6.60			5.8	
Heated pH 9		6.20			5.5	

[a]Heated at 120 C for 30 min.
[b]Sugar concentrations - 0.06 M.
[c]Urea - 8 M.
[d]Calcium - 0.3 M.
[e]Isotonic saline - 0.15 M.
[f]Demonstrated coagulation upon heating.

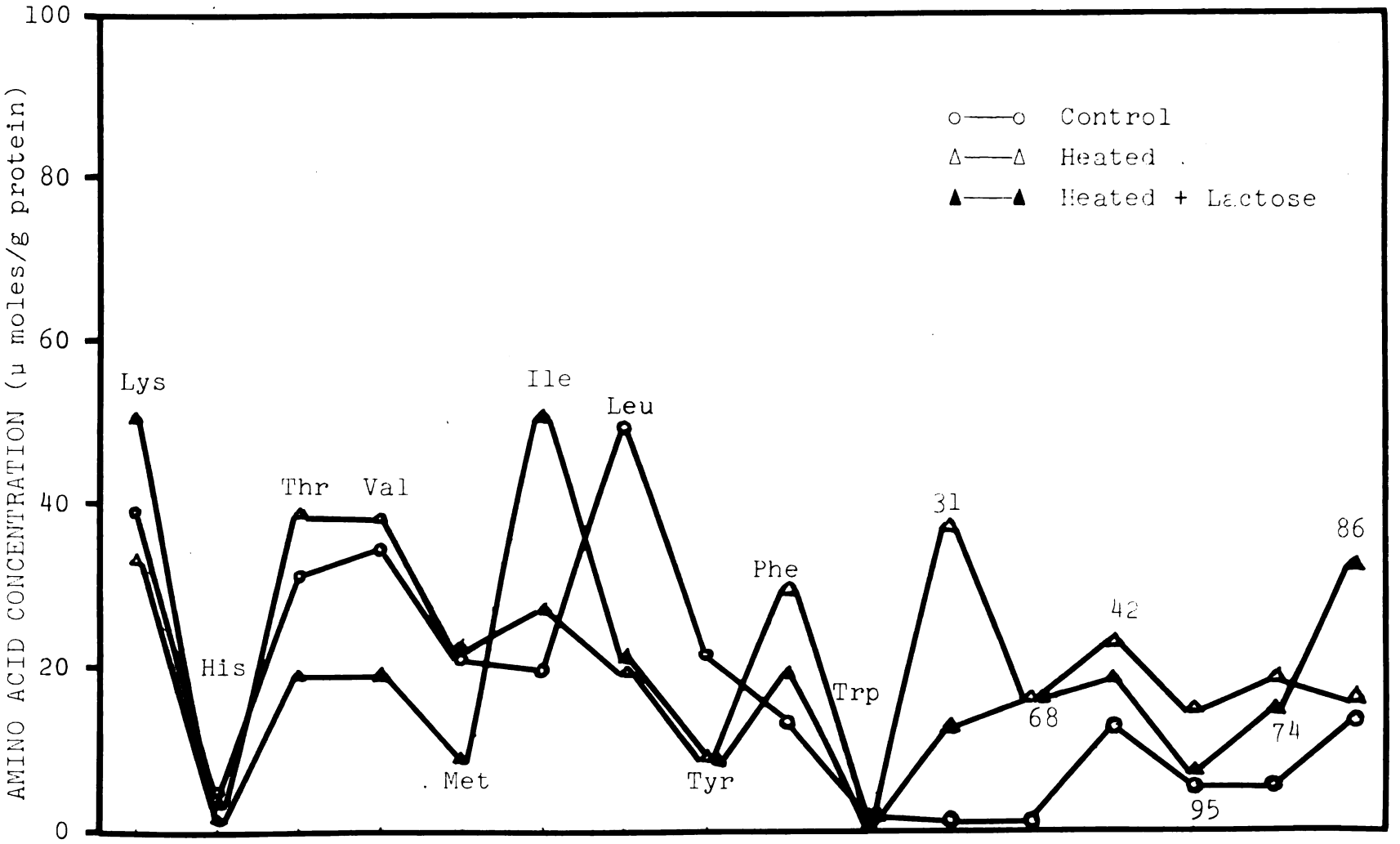

Figure 2.--Free essential amino acid and peptide profiles in pepsin-pancreatin digest of casein and heat-treated casein.

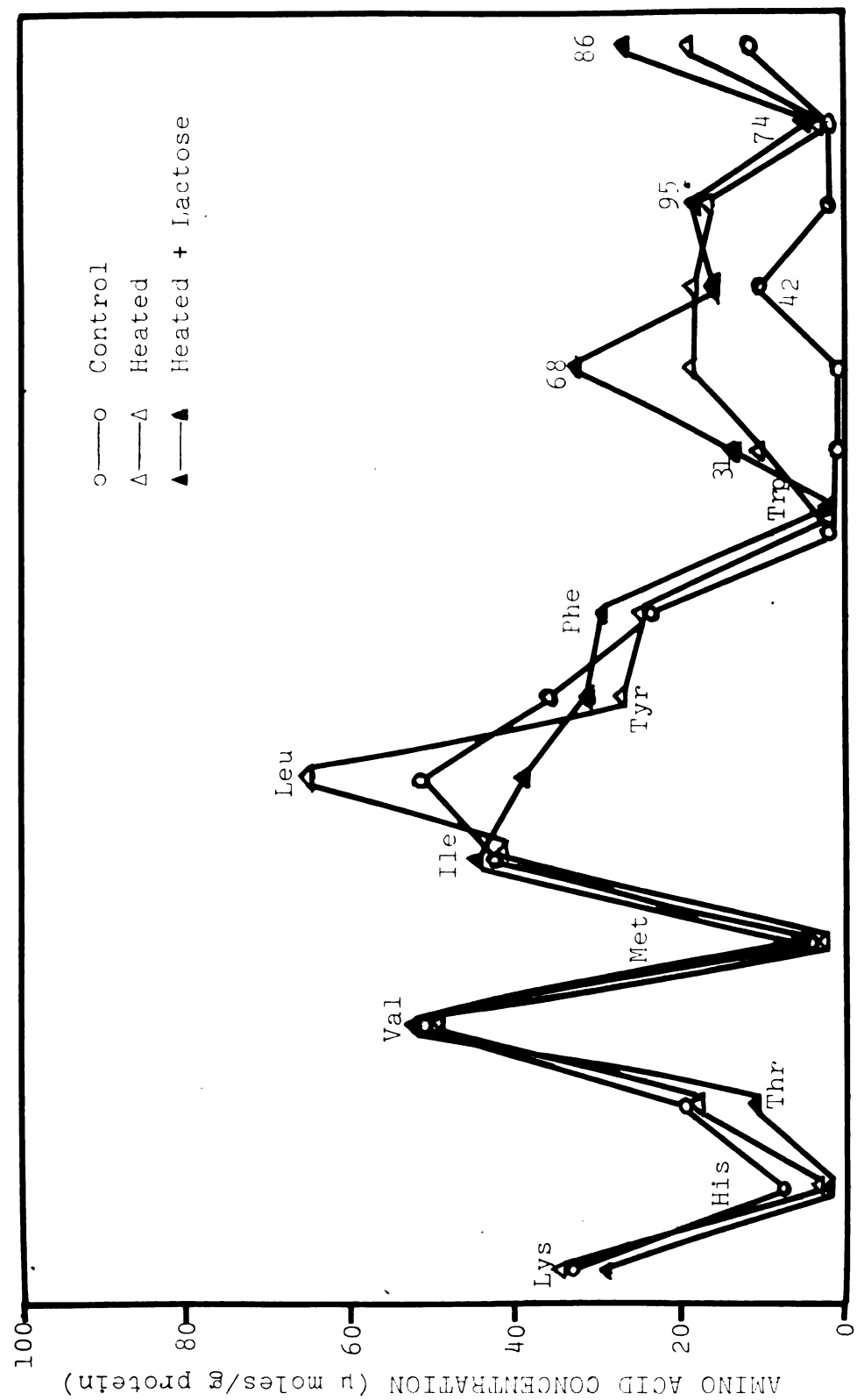

Figure 3.--Free essential amino acid and peptide profiles in pepsin-pancreatin digest of α_s-casein and heat-treated α_s-casein.

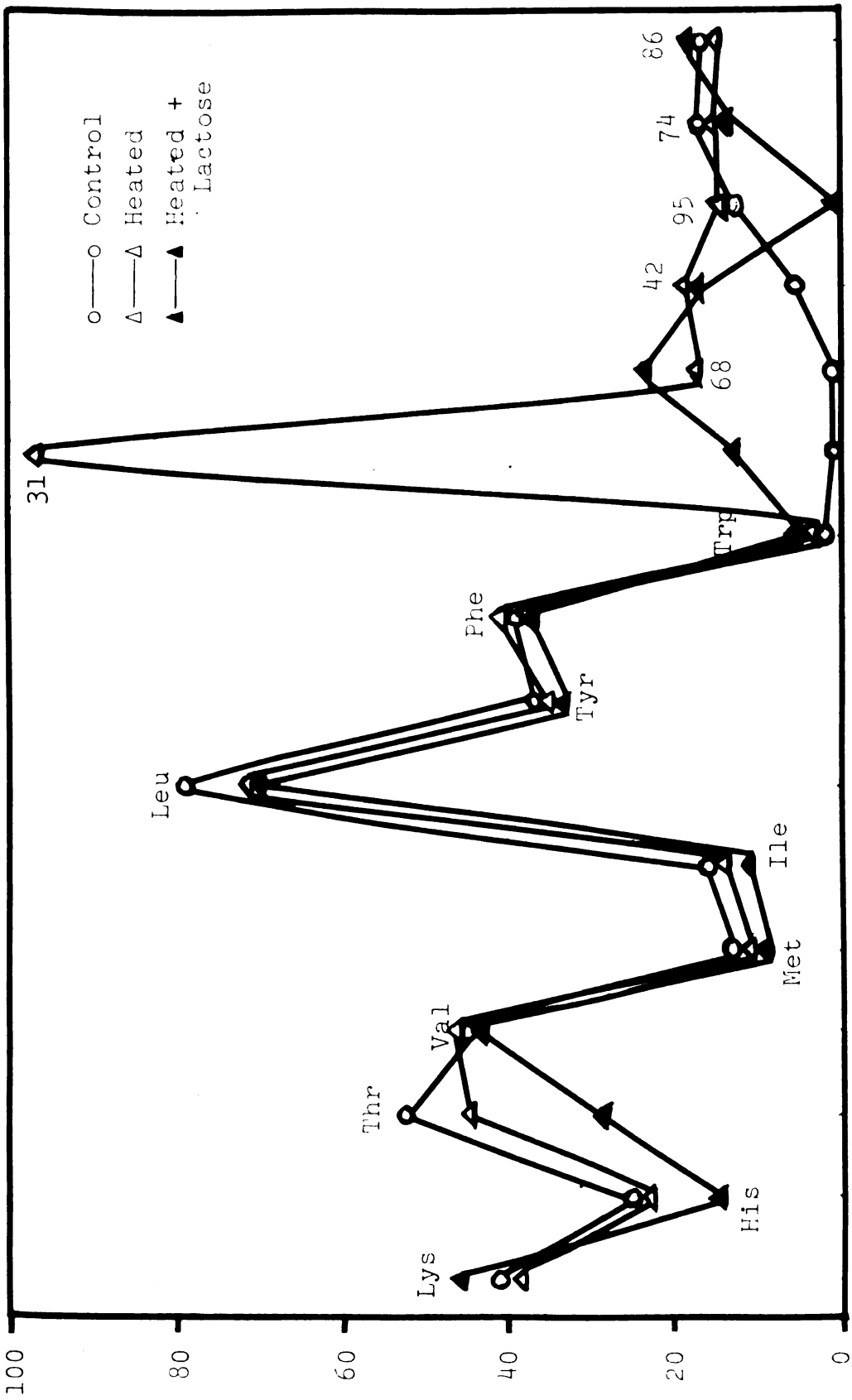

Figure 4.--Free essential amino acid and peptide profiles in pepsin-pancreatin digest of k-casein and heat-treated k-casein.

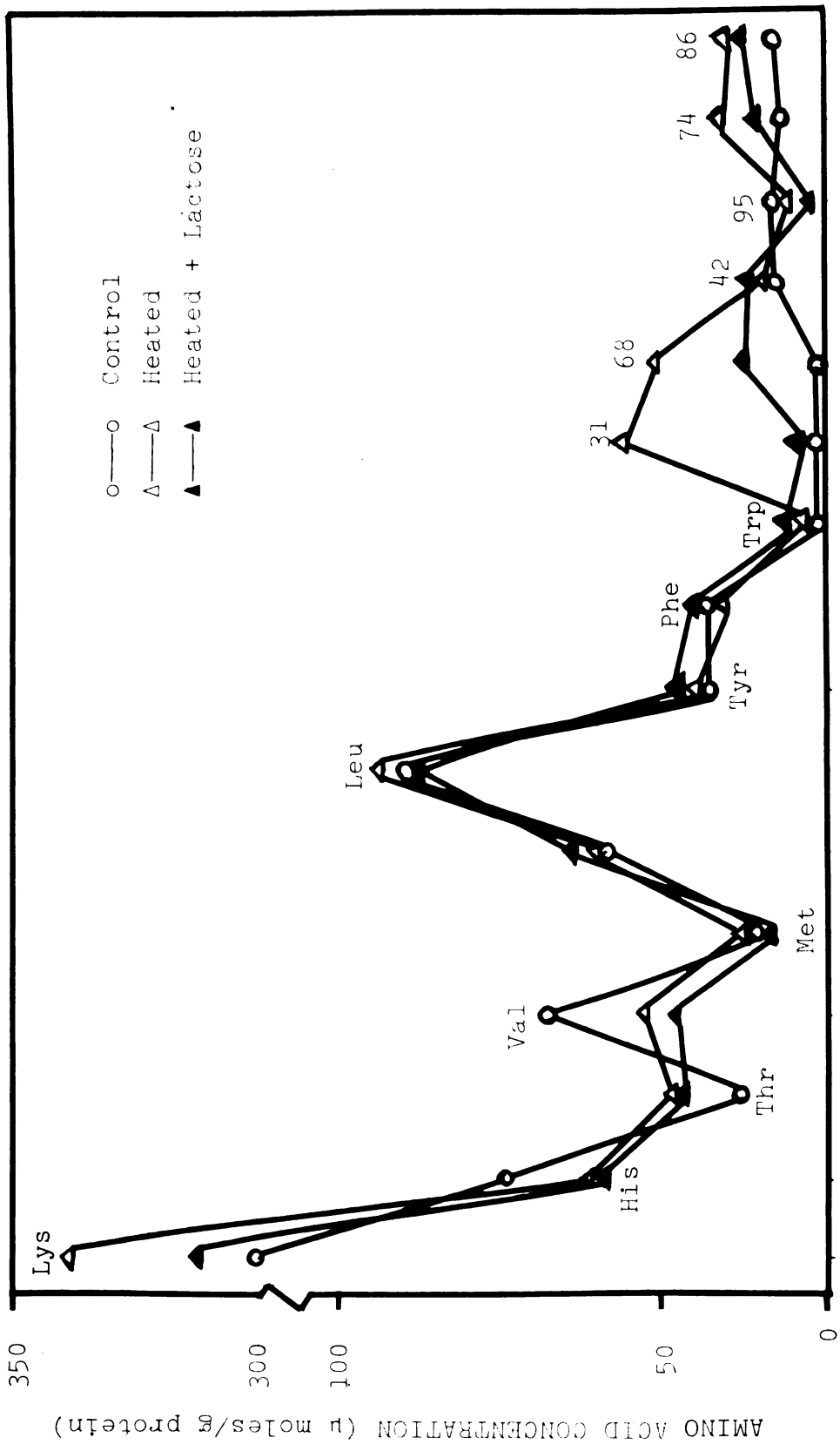

Figure 5.--Free essential amino acid and peptide profiles in pepsin-pancreatin digest of β-lactoglobulin and heat-treated β-lactoglobulin.

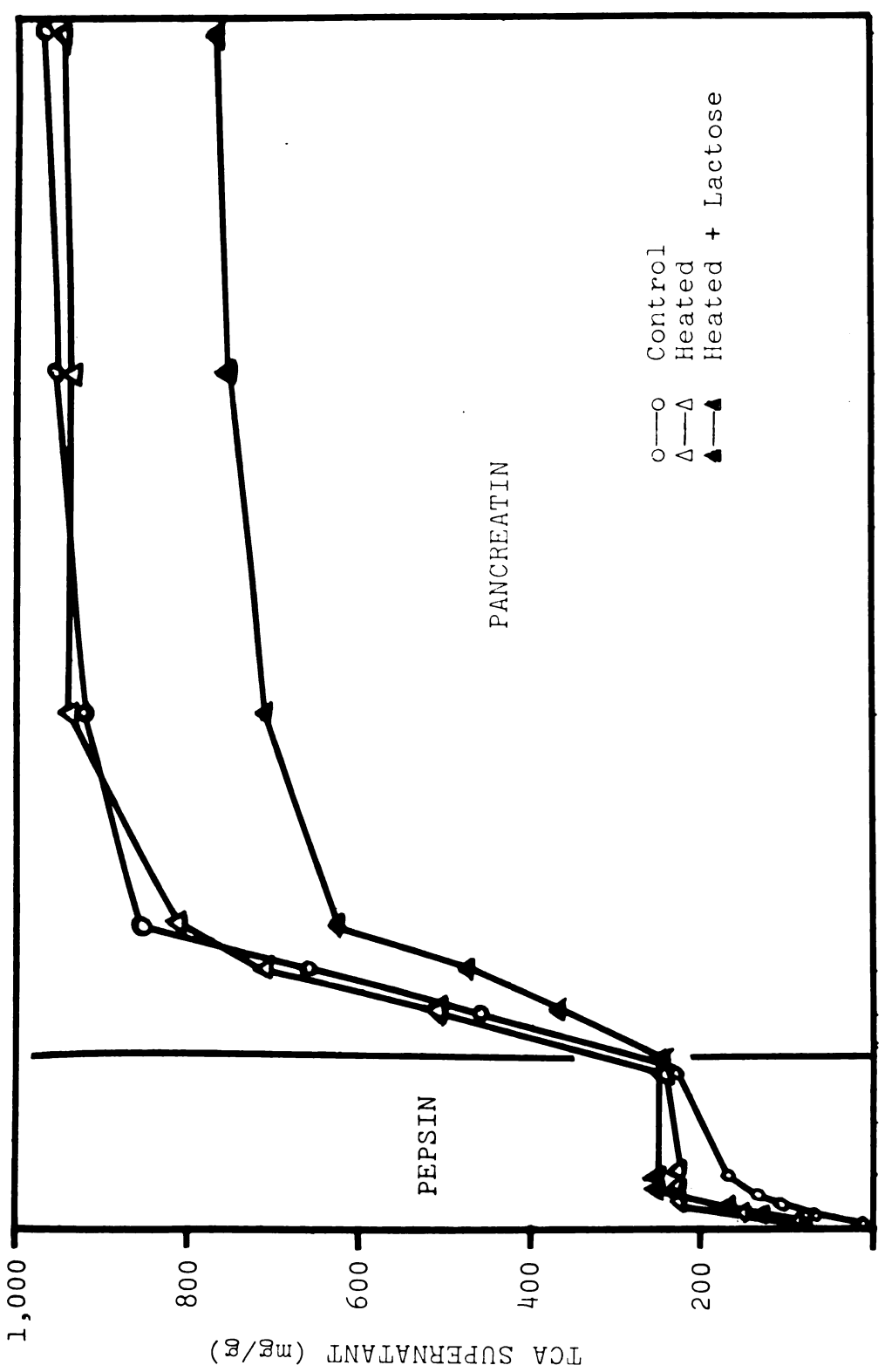

Figure 6.--Proteolysis during pepsin-pancreatin digest of casein measured by trichloroacetic acid-soluble protein.

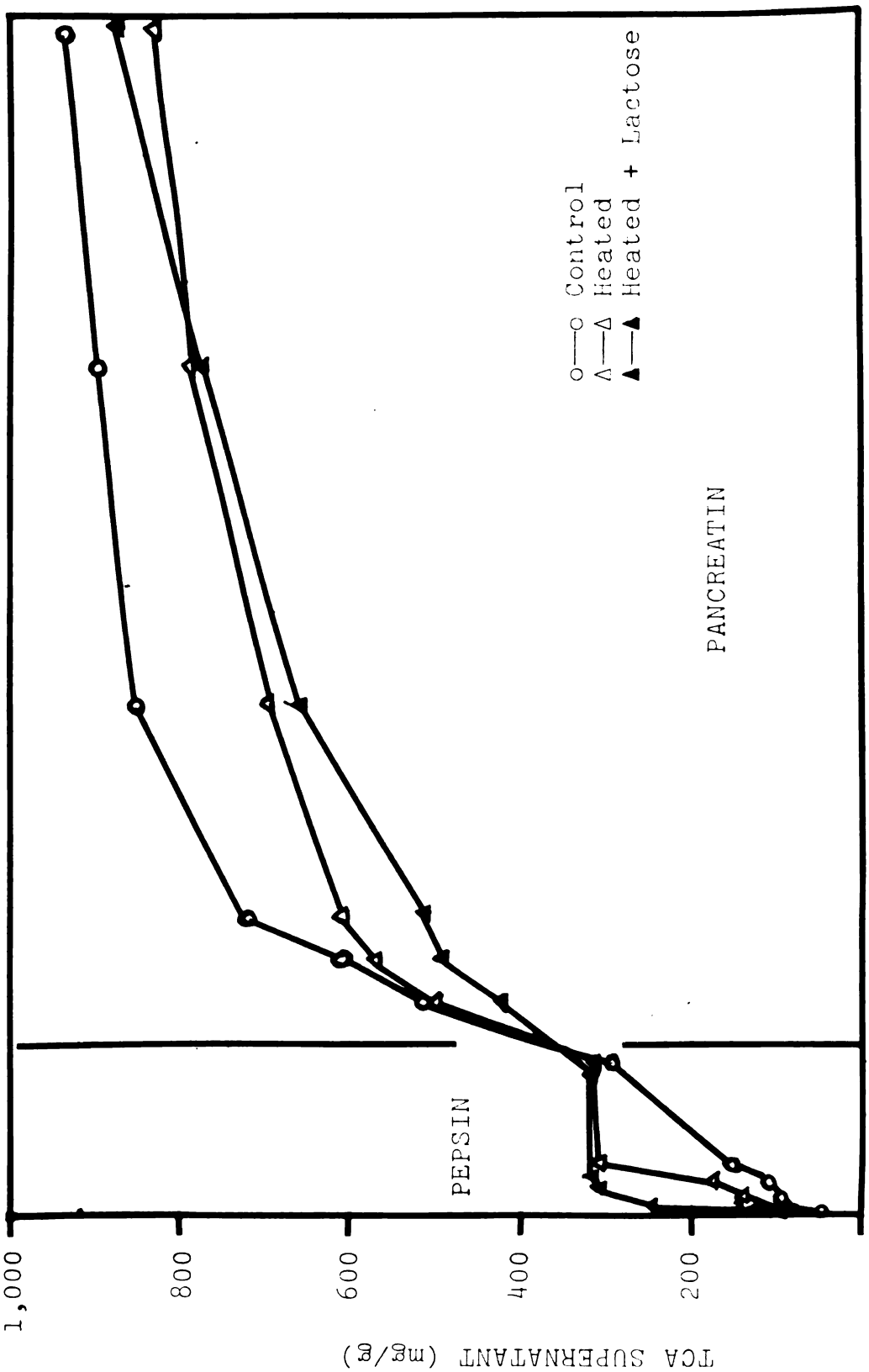

Figure 7.--Proteolysis during pepsin-pancreatin digest of α_s-casein measured by trichloroacetic acid-soluble protein.

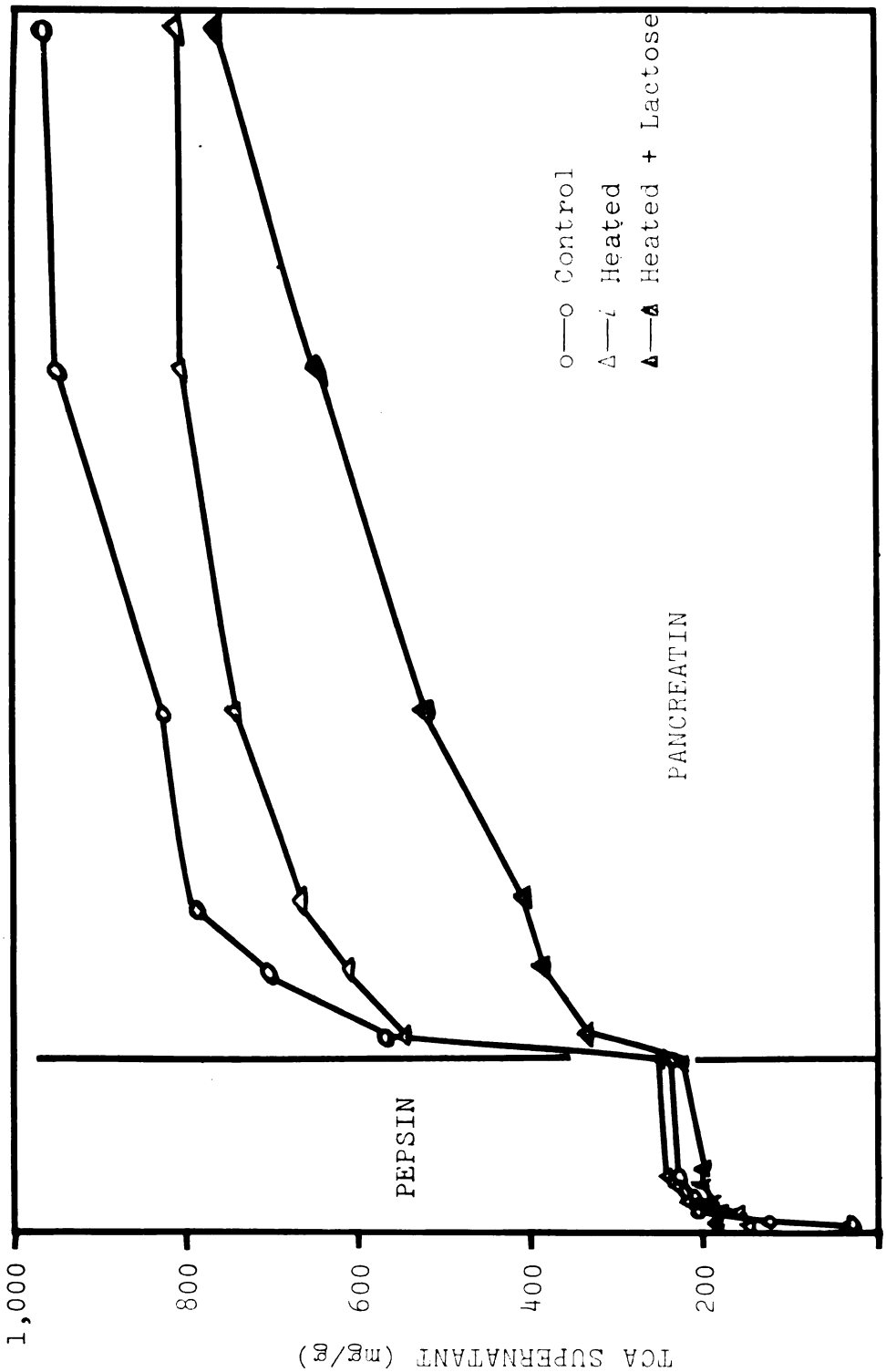

Figure 8.--Proteolysis during pepsin-pancreatin digest of k-casein measured by trichloroacetic acid-soluble protein.

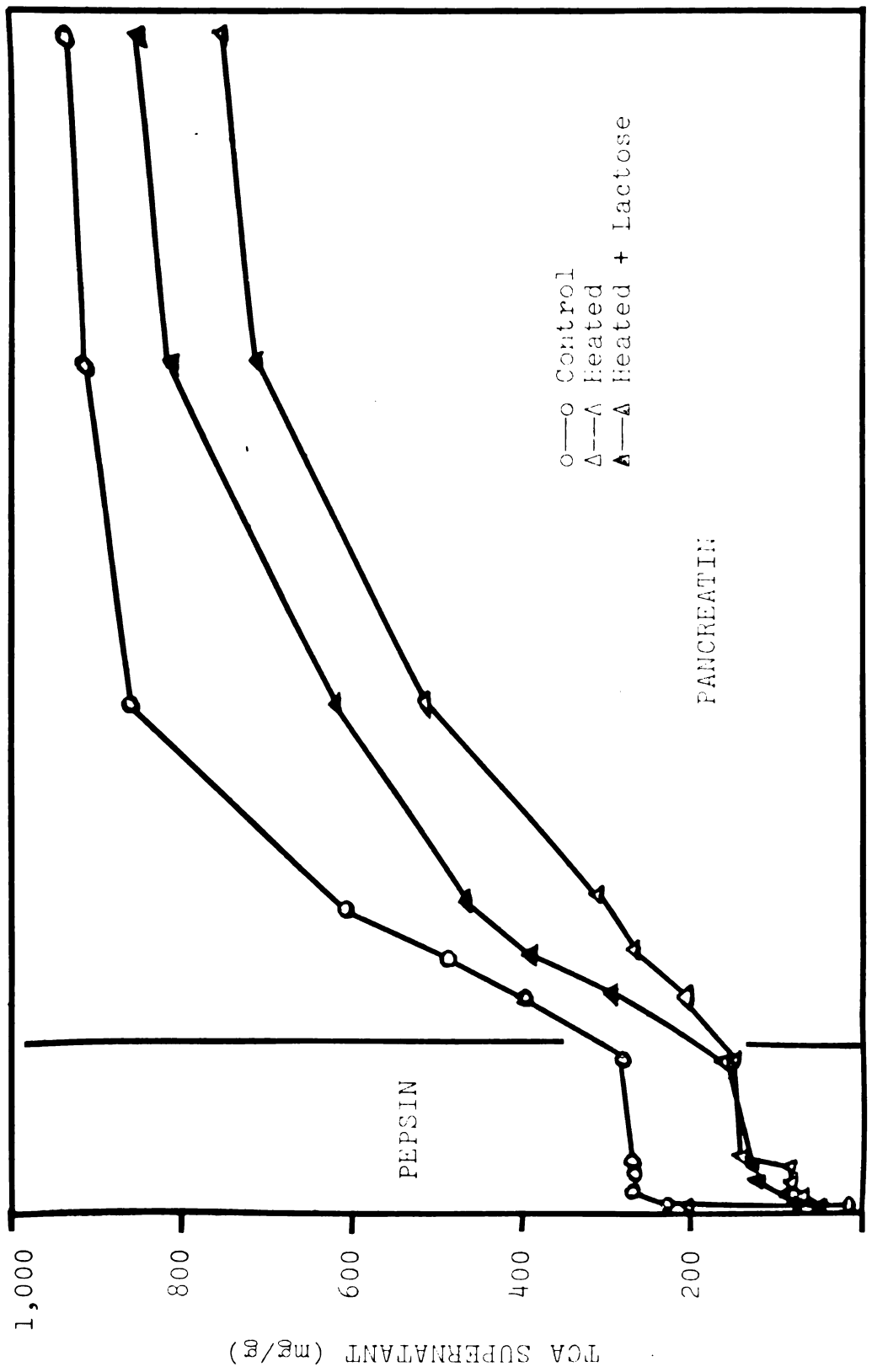

Figure 9.--Proteolysis during pepsin-pancreatin digest of β-lactoglobulin measured by trichloroacetic acid-soluble protein.

DISCUSSION

Milk Protein Preparations

Amino Acids

The amino acid contents contained in Table 2 for milk protein preparations indicate that basically the amino acid contents verify those published for protein fractions. Differences between amino acid contents from this study and those previously published represent variations in preparative techniques, analytical methods and inherent variation in milk. Particular amino acid differences are obvious for casein. The results reported by Block and Weiss (1956) represent analyses performed microbiologically, and the results for this study were analyzed chemically.

Characteristics of Protein Preparations Upon Heat Treatment

The formation of color during heat treatment of proteins can be associated with chemical characteristics of the individual proteins. The lack of color change with heat treatment of α_s-casein may be a ramification of the disordered configuration, lack of internal disulfide linkages for interchange and minimal aggregation tendencies

at the treatment temperature and pH. Swaisgood and Timasheff (1968) reported that at pH values greater than 7, low ionic strength (to 0.02) and protein concentration less than 10 g per liter, α_s-casein aggregation was favored at room temperature.

The color formation of k-casein, in contrast, was not changed significantly by the presence of lactose during heat treatment. Color formation with heat treatment of k-casein suggests reaction of the carbohydrate moiety of the molecule as well as complex formation resulting from the interactions of the internal disulfide linkages. The color formation observed after β-lactoglobulin was heated both with and without lactose was more pronounced than casein preparations and of a particular gray-tan color. The heated β-lactoglobulin color change may be partially attributable to complex formation of the type described by Trautman and Swanson (1959) or by the disulfide interactions suggested by Morr and Josephson (1968). The β-lactoglobulin susceptibility to coagulation, particularly during heat treatment in the presence of ionic calcium, has been reported by Rose (1962) as well as by Morr and Josephson (1968). The heat induced changes in β-lactoglobulin indicated by color change can be associated with unique characteristics upon enzymatic hydrolysis, particularly the rate of proteolysis observations reported in Table 16.

Pepsin-Pancreatin Digest of Proteins and Heat-Treated Proteins

Amino Acids and Peptides

The free amino acid contents of individual protein digests were indicative of enzymatic release characteristic for each protein, amino acid and treatment. The complexity of possible combinations and their significance makes examination of the amino acid data by several methods desirable.

The amino acids lysine and arginine are significant in that they represent the cleavage sites for trypsin (Hill, 1965). The lysine and arginine liberated by enzymatic proteolysis are expressed as percentages of the original quantities for each milk protein in Tables 3 through 6, and no consistent reduction in release rates can be associated with heat treatment. The lysine-arginine amounts released from casein were greater after heat treatment in the presence of lactose than unheated casein, but no change was associated with heat treatment without lactose. α_s-Casein demonstrated a decrease in freed lysine-arginine quantities when heated, consistent with the observations by Bujard et al. (1967) for milk proteins.

The free lysine-arginine contents from k-casein and β-lactoglobulin hydrolysates increased markedly after heat treatment. Heat treatment of k-casein and

β-lactoglobulin in the presence of lactose resulted in free lysine-arginine contents similar to unheated preparations. This suggests that heat denaturation may have made the cleavage sites more exposed and susceptible to complexes of this type suggested by Donoso et al.(1962) in the presence of lactose. The complexes they described formed a stable condensation bond between the reducing sugar components and amino groups. The carbon-nitrogen linkage formed was not hydrolyzable by digestive enzymes.

The enzymatic release of amino acids other than lysine and arginine was likewise individually different with preparation and treatment variation to make a single basis for expression desirable. Interpretive calculations have been proposed by several authors (Oser, 1959; Sheffner et al., 1956; Block and Mitchell, 1950) which provide correlation with animal studies. The manipulation used by Sheffner et al. (1956) as adapted by Akeson and Stahmann (1964) was used. The pepsin-pancreatin indices are logarithmic-geometric averages of free essential amino acid contents described as a ratio to egg protein under similar conditions. The pepsin-pancreatin indices for milk protein preparations are contained in Table 17. The pepsin-pancreatin essential amino acid index for casein (78) was in agreement with that reported by Akeson and Stahmann (1964) for casein. These results were greater than the value of 69 originally reported by

Sheffner et al. (1956), but their results were for a commercially dried preparation. A study conducted using humans as subjects by Hawley et al.(1948) led to an egg protein index of 73 for casein. The pepsin-pancreatin amino acid index therefore provides a correlary with animal studies.

Animal studies were not possible with the protein preparations in this study. The primary deterrent was the necessity for concentration of the protein preparations to provide a diet containing 8 to 10% protein. Protein concentration provides conditions favorable to, for example, aggregation for α_s-casein (Swaisgood and Timasheff, 1968); loss of free amino nitrogen in stored high-moisture milk powder (Lea, 1948); and β-lactoglobulin complex formation in evaporated milk (Trautman and Swanson, 1959). Another reason that animal studies were not conducted was that the heat treatment, 120 C for 30 min, was mild in comparison to many common processing conditions. It is therefore questionable that the differences would be significant, if measurable. Fricker (1964) was unable to demonstrate differences related to high temperature short time heat-treated milk in extensive feeding trials. The quantity of milk that would be required to prepare sufficient quantities of proteins for animal feeding was also a limitation. Zittle and Custer (1963) reported a yield of 12% for k-casein; however the yields in this study were approximately 3%.

The pepsin-pancreatin essential amino acid indices reported in Table 17 represent composites of free amino acid contents for each protein. The component amino acids can best be discussed relative to the essential amino acid index for individual model systems.

Casein.--The essential amino acid pepsin-pancreatin index for casein was lower after heat treatment (68) than unheated (78) and was considerably less (46) after heat treatment in the presence of lactose. Three essential amino acids were associated with the changes observed; histidine, threonine and leucine. Even though isoleucine and lysine were liberated in larger amounts from heat-treated casein, their contents are not reflected in the index since their quantities were greater than those in the reference protein.

The peptides from the pepsin-pancreatin digest of casein reported in Table 7 have a relationship to the pepsin-pancreatin index. The peptide quantities vary inversely with the pepsin-pancreatin index. Peptides designated 31 and 86 quantities are greater from the digest of casein heated in the presence of lactose corresponding to decreases in the amino acids; histidine, threonine and leucine. The association between peptides 31 and 86 and the essential amino acids indicates that these peptides may contain histidine, threonine or leucine.

Non-essential amino acids are not considered in the pepsin-pancreatin index; however their release by enzyme hydrolysis is pertinent. The amino acids aspartic acid, glutamic acid, glycine and alanine were released in smaller amounts after heat treatment with added lactose. The enzymatic liberation of non-essential amino acids corresponds to the pepsin-pancreatin index and reflects less complete proteolysis after heat treatment, especially with added lactose, than for unheated casein.

Casein, as a mixed protein, was unique among the proteins studied, and the inconsistent liberation of amino acids by enzymatic proteolysis can be noted in the amino acid profiles in Figure 2. In contrast, the amino acid profiles for more homogeneous preparations; α_s-casein, Figure 3; k-casein, Figure 4; and β-lactoglobulin, Figure 5; are more consistent in free amino acid contents of digests associated with heat treatments.

α_s-Casein.--The pepsin-pancreatin indes for α_s-casein was analogous to casein in magnitude and reduction associated with heat treatment, from 80 for unheated to 67 for heated protein. The similarity for heated α_s-casein to casein is carried through to amino acid differences. Histidine and threonine were less in the heated than unheated α_s-casein digest in amounts similar to comparable measurements for casein digests. Likewise, the peptides 31 and 86 were present in larger amounts in the heated

α_s-casein digest than the unheated, to suggest contents of histidine and/or threonine.

α_s-Casein heated in the presence of lactose resulted in a pepsin-pancreatin index slightly higher (70 to 67) than that for the heated protein. The amino acid index increase associated with heat treatment in the presence of lactose was observed only for α_s-casein. Also significant was the outstanding amount of peptide 68 in the digest of α_s-casein heated in the presence of lactose. Comparison of essential amino acids from casein and α_s-casein in pepsin-pancreatin digests indicates that the amino acids that can be associated with the striking differences are threonine and histidine. The outstanding and unique increase in peptide 68 in the digest of α_s-casein heated in the presence of lactose was associated with a smaller amount of arginine, suggesting that arginine may be a component of peptide 68.

k-Casein.--A significant increase in the pepsin-pancreatin index from 64 for unheated to 79 for heated was derived for k-casein. The pepsin-pancreatin index of k-casein heated in the presence of lactose (73) was also greater that that for the unheated preparation and only slightly less than that for k-casein heated alone. The more extensive k-casein proteolysis associated with heat treatment is possibly attributable to configuration characteristics after heat denaturation. A similar

phenomenon was reported by Kakade and Evans (1966b) for navy bean protein. Mitchell and Block (1946) proposed that heat rendered the amino acids of ordered proteins more available without changing actual contents of the amino acids.

The amino acids released from heated k-casein preparations in greater quantities than from unheated preparations were phenylalanine, valine and lysine, as reported in Table 5. The amounts of histidine and threonine were less in digests of heated k-casein than unheated, in magnitudes similar to the other casein hydrolysates. The quantities of histidine and threonine present in the hydrolysates of k-casein preparations were greater than amounts in the index protein and, as a result, the quantities were not limiting and did not influence the pepsin-pancreatin index. The compensation for non-limiting amino acids represents an inherent difficulty in index evaluation of amino acid quantities. The validity of the index technique has been established, however, as a proper compensation (Block and Mitchell, 1946; Oser, 1959; Sheffner, et al., 1956).

The peptide distribution for digests of k-casein preparations was unique among proteins examined, as peptide 31 was outstanding particularly from heated protein. The contents of histidine and threonine associated with the peptide 31 suggest that histidine and/or threonine may be components as has been noted for casein and α_s-casein.

The free arginine in digests of k-casein proteolysis preparation was 30% greater from the heated than the unheated preparations. Since arginine is one of the tryptic cleavage sites, the increased quantity may implicate heat-induced availability associated with denaturation.

β-Lactoglobulin.--The pepsin-pancreatin indices for β-lactoglobulin were not significantly different for unheated or heated preparations. The inability fo discriminate between heated and unheated β-lactoglobulin can be attributed to the amounts of essential amino acids in excess of the index protein. The same observation was noted for heated k-casein hydrolysates. The amounts of lysine and arginine freed by hydrolysis in Table 6 suggest that heat treatment may cause denaturation to make the tryptic cleavage sites more readily available. The quantities of lysine and arginine are 5 to 15% greater from heated preparations, with and without lactose, than from control preparations.

The amounts of free tyrosine in the pepsin-pancreatin hydrolysates for β-lactoglobulin in Table 6 are all greater than the total composition released by acid hydrolysis. The descrepancy may represent an analytical inconsistency and a trait of hydrolysis. The losses of tyrosine upon acid hydrolysis may be as great as 10% suggested by Block and Weiss (1946). Pepsin-pancreatin digests were not subjected to this loss. Tyrosine is one

of the cleavage sites for pepsin (Hill, 1965). The conformation of β-lactoglobulin may make many of the tyrosine sites available for pepsin cleavage. The efficient pepsin cleavage of β-lactoglobulin may be associated with increased degree of tryptic digest after pepsin treatment as observed by Linderstrøm-Lang, et al. (1938).

The free non-essential amino acids from β-lactoglobulin digests were present in larger amounts after heat treatment than in unheated protein. An important exception is the content of cysteine, which was freed in much smaller quantities from heated β-lactoglobulin. The decrease in cysteine content associated with heat treatment indicates the lability of the sulhydryl-disulfide group and the reactivity described by Morr and Josephson (1968). A similar decrease was not noted from digests of heated k-casein, although k-casein also contains internal disulfide linkages. This may indicate that the same secondary structural characteristics suggested by Tanford et al. (1962) for β-lactoglobulin do not exist for k-casein.

Peptides.--The peptides observed in the pepsin-pancreatin digests of each protein preparation (Table 7) represent relative quantities since peptide standards are not available. The disappearance of histidine and threonine was associated with increased amounts of peptide 31, implying that one or both of these amino acids may be constituents. Mobility in the methanol/water/pyridine

solvent system indicated behavior similar to that of histidine and threonine, as peptide 31 moved between the two amino acids. Peptide 86 was also increased in digests of heated proteins. The mobility of peptide 86 was indicative of an aliphatic amino acid.

Proteolysis During Pepsin-Pancreatin Digestion

The pepsin-pancreatin proteolysis of milk protein preparations, monitored by trichloroacetic acid-soluble Lowry-Folin reactive material, resulted in a number of observations true for all preparations. The trichloroacetic acid supernatant protein was greater in heated protein preparations than for unheated samples before enzymatic proteolysis. Similar observations were reported by Ghadimi and Pecora (1963) for picric acid supernatants. The initial trichloroacetic acid-soluble protein may suggest disruption of the protein molecular configuration to either make a portion of each molecule soluble or a portion of the total number of molecules soluble.

Casein.--The pepsin-pancreatin proteolysis of casein monitored by trichloroacetic acid-soluble protein is described graphically in Figure 6. Pepsin proteolysis of casein reflects distinct differences between heated and unheated proteins. Peptic proteolysis of heated preparations proceeded to completion within a 1-hr period, while the unheated protein was more slowly hydrolyzed throughout

the 3-hr period. Pancreatin hydrolysis of casein, unheated and heated, proceeded at essentially the same rate. The casein preparation heated in the presence of lactose was hydrolyzed by pancreatin considerably less rapidly with the final amount of hydrolyzed protein 20% less than preparations otherwise treated. The observations for casein are essentially consistent with pepsin-pancreatin index measurements.

α_s-Casein.--Peptic proteolysis of α_s-casein (in Figure 7) was analogous to that for casein. Heated protein preparations had attained the final level of trichloroacetic acid-soluble protein within the first hour. A larger portion of the protein was solubilized by pepsin in the case of α_s-casein (28 to 31%) than in the case of casein (23 to 24%). The pancreatin hydrolysis rate for unheated α_s-casein was similar to casein; however a much more pronounced heat treatment influence was obvious with heated α_s-casein which was less extensively hydrolyzed. The response to heat treatment may be associated with the disorganized structure of α_s-casein reported by Herskovits (1966). The amounts of α_s-casein solubilized by pepsin-pancreatin hydrolysis were consistent with the pepsin-pancreatin indices for α_s-casein.

k-Casein.--The k-casein pepsin-pancreatin proteolysis monitored by trichloroacetic acid-soluble protein is described graphically in Figure 6. The peptic proteolysis

for k-casein preparations monitored by trichloroacetic acid-soluble protein was not as extensively influenced by heat treatment as were casein and α_s-casein. The degree of hydrolysis by pepsin had attained the final level for all preparations after a 1-hr exposure. The extent of pancreatic proteolysis of k-casein by heat treatment and heat treatment in the presence of lactose was reduced by 19 to 20% from unheated k-casein. The reduction in pancreatic proteolysis associated with heat treatment for k-casein was not in agreement with the pepsin-pancreatin amino acid index which was higher for heated than unheated proteins. The difference between the amino acid index and trichloroacetic acid-soluble protein is due to the released amounts of threonine and histidine from k-casein which are not limiting when compared to the index egg protein.

β-Lactoglobulin.--The pepsin-pancreatin proteolysis of β-lactoglobulin monitored by trichloroacetic acid-soluble protein is indicated in Figure 9. Peptic digest of β-lactoglobulin did not proceed after the first hour in any case. Proteolysis of heated protein was only half as extensive as was peptic hydrolysis of unheated β-lactoglobulin. Heat-induced complex formation of the type suggested by Trautman and Swanson (1959) or the molecular associations described by Tanford et al. (1962) may have been partially responsible for the less extensive

proteolysis of β-lactoglobulin. Pancreatic hydrolysis of β-lactoglobulin, on the contrary, was more extensive than unheated protein for heated preparations and still more extensive for the protein heated with added lactose. The pepsin-pancreatin amino acid index for β-lactoglobulin did not indicate similar amounts of amino acid release as the trichloroacetic acid-soluble protein. The amino acids threonine and histidine were not limiting, as was the case with k-casein.

Enzymatic Proteolysis During Gel Filtration

Enzymatic proteolysis of heated proteins during gel filtration provides a rapid method for estimation of the degree of protein hydrolysis. Broad interpretation of the results derived must be approached with caution, as the specificity of the enzyme may be measured rather than treatment differences (Ford and Salter, 1964). Zebrowska (1968) confirmed the precaution suggesting that only "broad similarity" exists between the *in vitro* measurements from gel filtration proteolysis and those obtained with rats. Several advantages are ascribed to the technique of enzymatic proteolysis during gel filtration, including: minimal transamination, stepwise proteolysis and proteolysis under realistic conditions insofar as absorption of amino acids is concerned.

Total Protein Distribution

The protein content of fractions separated after enzymatic proteolysis during gel filtration of milk proteins are reported in Table 9. The variable protein amounts eluted as intact protein indicate that a general statement to describe influence of heat treatment on susceptibility to tryptic cleavage is not possible.

Casein and α_s-casein heat-treated preparations contained less protein in the combined peptide and amino acid fractions (Fractions 2 and 3) than the unheated protein. The protein preparations containing disulfide linkages, k-casein and β-lactoglobulin, demonstrated a dissimilar tendency, as the amount of protein in the amino acid and peptide fractions from heated proteins was greater than or equal to the amount from unheated proteins. The stimulation of proteolysis by heat treatment of k-casein and β-lactoglobulin was previously noted in pepsin-pancreatin proteolysis and may be associated with heat denaturation.

Trichloroacetic Acid Supernatant Protein

Trichloroacetic acid-soluble Lowry-Folin reactive material in fractions collected after gel filtration proteolysis provides an indication of the degree of tryptic hydrolysis. The trichloroacetic acid-soluble protein contents reported in Table 10 demonstrated the

same characteristics described for the total protein content of the gel-filtration proteolysis fractions. The trichloroacetic acid-soluble portion of the intact protein (Fraction 1) showed increases attributable to heat treatment of 20 times for casein and 3 to 4 times for α_s-casein. On the contrary, the intact protein after gel filtration proteolysis of heated k-casein was lower than the unheated protein, and an increase of only 24% was observed after heat treatment with added lactose. The trichloroacetic acid-soluble portion of the intact protein fraction from β-lactoglobulin gel filtration proteolysis was approximately half the amount from control protein in the heated preparations.

The trichloroacetic acid-soluble protein from heated preparations before hydrolysis was higher than from unheated preparations suggesting that dissociation of complexes and some heat-induced peptide-linkage cleavages may occur.

Reaction with 1-Fluoro-2, 4-Dinitrobenzene

The reaction of protein with FDNB to measure available lysine has gained acceptance since the method was published by Carpenter (1960). The milk proteins in this study were evaluated by reaction with FDNB before and after heat treatment. The protein fractions from gel filtration proteolysis were also reacted with FDNB. The

initial quantities of ε-amino-DNP-lysine for milk proteins in Table 10 are slightly greater (15 to 23%) than the results for milk protein reported by Bujard et al. (1961), but similar to those reported by Lea and Hannan (1950). Lysine inactivation of 20% was described in a report by Bujard et al. (1967) for evaporated milk (heated in condensed state at 113 C for 15 min). Casein results showed a 4% decrease in lysine associated with heat treatment in the presence of lactose in this study. Pepsin-pancreatin digest of the evaporated milk described by Bujard et al. (1967) led to a lysine availability of 74% compared to 59% for casein in this study. The descrepancy in lysine contents is probably associated with three differences in sample preparation. The evaporated milk of Bujard et al. (1967) contained the entire milk protein at a concentration of 8.9% protein. Their conditions were more advantageous to protein-carbohydrate complex formation than the relatively low concentration and homogeneous systems in this study. Heat treatment at 113 C for 15 min by Bujard et al. (1967) was considerably less than for proteins in this study (120 C, 30 min). The comparative results indicate greater apparent lysine loss with evaporated milk but fewer linkages resistant to hydrolysis. The third difference involves liberation of α-amino linkages during heat treatment. The trichloroacetic acid-soluble protein fractions have indicated that release of

soluble protein may be attributable partially to heat-induced peptide cleavage. The reaction of α-amino groups freed by heat-induced cleavage would tend to offset the loss of availability suggested by Bujard et al. (1967). Boctor and Harper (1968) have evaluated the FDNB method for protein evaluation in rats and contend that it is not suitable for lysine estimation in heat-treated proteins. Their contention was based on the large amounts of FDNB reactive compounds in the feces of rats.

Milk protein fractions collected after proteolysis during gel filtration (Table 10) contained in all cases more FDNB reactive material estimated as lysine than did the original protein. The observation of Boctor and Harper (1967) is substantiated by the contents of the gel-filtration hydrolysis fractions. Fecal material would reflect any FDNB reactive material not absorbed and would include α-amino groups in addition to ε-amino lysine.

Quantities of terminal amino groups noted in the fractions from proteolysis during gel filtration of unheated casein and k-casein were much larger than other protein preparations. The initial proteolysis during pepsin-pancreatin digest was rapid and was possibly a measurement of the same phenomenon. The FDNB reactive material in the three fractions derived from gel hydrolysis of β-lactoglobulin indicates more extensive hydrolysis after heat treatment, as was observed in the free amino

acids released by pepsin-pancreatin proteolysis. The reduced amounts of FDNB reactive materials observed in the fractionated effluents derived from α_s-casein heated in the presence of lactose suggest the formation of complexes not susceptible to tryptic cleavage.

Rate of Proteolysis by pH-Stat

The rates of proteolysis estimated by pH-stat monitored proton release provide a rapid and sensitive method for evaluation of enzymatic proteolysis. The methods of expression, substrate concentration and specific activity as an expression of reaction velocity, provide duplicate bases for discussion of the observation. Rapid and efficient proteolysis is described by a maximum rate at minimum concentration.

Equal Cleavage Sites

Preliminary examination of milk proteins resulted in kinetic differences between individual protein preparations equated on the basis of molar concentration. The hypothesis that differences in proteolysis rate was governed by the number of cleavage sites rather than molar concentration was evaluated. The substrate concentrations and specific activities for milk protein preparations with equal lysine and arginine concentrations are reported in Table 12. The dissimilarity of rates of proteolysis suggests that chemical and physical composition

are more responsible for enzymatic proteolysis rate than number of cleavage sites.

Chemical Denaturing Agents

The basis for discussion of treatments evaluated by rates of enzymatic proteolysis must be established by description of fundamental milk protein measurements. The rates of proteolysis are enumerated for milk protein systems in Tables 13 through 16.

Casein specific activity results were less after heat treatment (2.2×10^{-3} mM/min) than untreated (2.8×10^{-3} mM/min) and reduced to 1.6×10^{-3} mM/min after heat treatment with added lactose. The casein proteolysis rate results can be related to pepsin-pancreatin indices for corresponding casein preparations; control - 78, heated - 68, and heated in the presence of lactose - 46.

The α_s-casein rate measurements are similar to the corresponding treatment values observed for the pepsin-pancreatin amino acid index. The α_s-casein relationship is not as straightforward as was casein because the substrate concentration optima were reduced for α_s-casein.

The specific activity for k-casein was the same for heated as unheated preparations. The k-casein proteolysis rate after heat treatment with added lactose was greater than the unheated protein corresponding to the pepsin-pancreatin indices for k-casein preparations.

The specific activity measurements for β-lactoglobulin were the same for control, heated, and heated in the presence of lactose. The uniformity in specific activity for β-lactoglobulin corresponds to similar uniformity in pepsin-pancreatin indices for β-lactoglobulin preparations. Substrate concentration optima suggest previously noted heat denaturation and complex formation since a decrease in concentration from 4.08 to 1.11 x 10^{-3} M resulted from heat treatment denaturation. A concentration optimum of 2.50 x 10^{-3} M for β-lactoglobulin heated with lactose suggested possible lactose-protein complex formation.

Urea.--Casein and β-lactoglobulin treated with 8M urea were enzymatically hydrolyzed at a more rapid rate than the corresponding untreated proteins. Casein and β-lactoglobulin only were treated with urea as α_s-casein and k-casein were so treated in fractionation. Casein demonstrated a two-fold increase in specific activity associated with heat treatment and a four-fold increase when heat treated with added lactose. β-Lactoglobulin, on the other hand, showed an increase of 25% in specific activity due to heat treatment. β-Lactoglobulin heated in the presence of lactose was hydrolyzed at a rate equivalent to 35% of the unheated protein. Concentration optima for β-lactoglobulin after urea treatment were less tha 1/10 of the untreated proteins, suggesting denaturation.

Since the same difference in concentration optima were not obvious for casein, the results suggest that dissociation of casein by urea was reversible but was not for β-lactoglobulin.

Mercaptoethanol.--2-Mercaptoethanol is a denaturing agent used for disruption of disulfide linkages as reported by Anfinsen and Haker (1961). The rate of proteolysis measurements of 2-mercaptoethanol-treated α_s-casein were the same as for untreated preparations. α_s-Casein contains no disulfide linkages (McKenzie, 1967) which probably can be associated with the lack of change in proteolysis rate attributable to 2-mercaptoethanol treatment. The specific activities for casein, k-casein and β-lactoglobulin unheated preparations were lower for 2-mercaptoethanol-treated than for untreated proteins. The reduced proteolysis rates after heat treatment suggest that the rearranged protein forms after denaturation led to less readily available cleavage sites. Heat treatment of 2-mercaptoethanol-treated casein and k-casein resulted in specific activities greater than untreated preparations; however the reverse relationship existed for β-lactoglobulin. The reduced rate of proteolysis for heated β-lactoglobulin suggested an occlusion of cleavage sites in the denatured protein. The rates of proteolysis for casein, k-casein and β-lactoglobulin after heat treatment with added lactose were considerably greater than untreated

preparations. Reassociation was apparently kept to a minimum by the presence of lactose, resulting in a readily hydrolyzable configuration.

Performic acid.--Hirs (1956) described the tryptic hydrolysis of ribonuclease after performic acid oxidation as approximately 1/10 its original quantity. Rate measurements monitored by pH-stat provide a somewhat different observation although not in conflict. The rates of proteolysis, described as specific activity, were greater after performic acid oxidation than for corresponding untreated preparations. The concentration optima were approximately one-fourth the original values for corresponding protein preparations. The combined phenomena suggest that irreversible denaturation occurred in a uniform amount not significantly influenced by heat. The irreversible denaturation may render a large protion of the original protein molecule available for hydrolysis, to conform with the observation by Hirs (1956). The influence of performic acid treatment on α_s-casein in a manner analogous to proteins containing disulfide linkages suggests that methionine may be involved in site availability since α_s-casein contains no disulfide linkages.

Calcium.--The presence of calcium ions resulted in the most pronounced change in rate of proteolysis for α_s-casein. The calcium sensitivity for α_s-casein described

by McKenzie (1967) resulted in reduction of specific activity for α_s-casein by 45% in unheated and heated samples and 25% in samples heated with lactose. Aggregation tendencies were indicated in α_s-casein preparations by higher concentration optima for calcium-treated proteins, particularly in combination with heat treatment. The specific activity for k-casein was not changed by calcium treatment, indicating the relative calcium stability reported by Zittle and Custer (1963). β-Lactoglobulin preparations demonstrated up to fivefold increases in specific activity associated with calcium addition. The most pronounced increases in β-lactoglobulin specific activity, three times control for heated and five times control when heated in the presence of lactose, were accompanied by visible coagulation of the protein. The coagulation of β-lactoglobulin may provide denatured conditions favorable to proteolysis.

Stabilization of trypsin by calcium has been described by Bergmeyer (1963). Trypsin stability brought about by the presence of calcium may have been responsible for a part of the increased proteolysis rates.

Sodium chloride.--Treatment of milk protein preparations with isotonic saline resulted in minimal changes in specific activity associated with heat treatment or heat treatment with added lactose. The milk protein molecules may have been stabilized or "salted in" by the presence of isotonic saline.

Pepsin pretreatment.--The influence of partial selective proteolysis before tryptic proteolysis rate evaluation was examined by pepsin pretreatment of milk protein preparations. Linderstrom-Lang (1938) observed that β-lactoglobulin was more susceptible to tryptic proteolysis after pepsin digest, which was verified by pH-stat measurement in this study. The action of pepsin has been compared to rennin by Fish (1957) making an analogy possible for pepsin predigestion. Porter (1964) reported that the action of rennin did not modify the behavior of casein upon tryptic hydrolysis. The change in specific activity of k-casein preparations was not large due to pepsin pretreatment to correspond with the contention set forth by Porter (1964). Proteolysis rate measurements for casein preparations reflected disruption by peptic digest, as rate increases of as much as four times the untreated preparations were observed. The specific activity measruements for α_s-casein preparations were substantially reduced for unheated and heated samples after pepsin predigestion. The reduction in tryptic digest rates may be indicative of extensive peptic proteolysis of α_s-casein resulting in insufficient amounts of the protein remaining for precise measurement.

pH treatment during heating.--Milk protein preparations were heated at pH 5 and at pH 9 before proteolysis rate measurement. The pH values represent a logical

pH range encountered in protein containing, heat-treated foods.

Heat treatment of casein and α_s-casein preparations at pH 5 resulted in reduction of proteolysis to levels less than could be measured. Heat treatment of casein at pH 5 followed by pepsin pretreatment led to specific activity slightly greater than untreated casein but at an insignificant concentration optimum. α_s-Casein after heat treatment at pH 5 had a specific activity one-tenth as great as unheated protein. Proteolysis of k-casein heated at pH 5 was enhanced, as reflected by a 26% increase in specific activity at a 15 times higher substrate optimum than unheated k-casein. β-Lactoglobulin heated at pH 5 was more rapidly hydrolyzed than the unheated protein, similar to the response by k-casein. The specific activity for β-lactoglobulin heated at pH 5 increased by 20%, and the concentration optimum was slightly more than double the unheated protein.

Casein preparations heated at pH 9 reflected a 10% reduction from unheated proteins in both specific activity and substrate concentration optima. The same casein preparations after pepsin pretreatment were not measurably hydrolyzed. The observations suggest change in micellar structure of casein. The hydrolysis sites were evidently available only after pepsin predigestion. α_s-Casein heated at pH 9 was also less rapidly hydrolyzed, with

reduction of specific activity to 25% of the control value and reduction of substrate concentration optimum to 32% of the unheated protein. k-Casein preparations heated at pH 9 did not demonstrate marked reduction in specific activity. The k-casein heated at pH 9 and then subjected to pepsin pretreatment was hydrolyzed more rapidly by trypsin. Heat treatment of β-lactoglobulin at pH 9 resulted in no significant change in rate of tryptic hydrolysis. β-Lactoglobulin heated at pH 9 was much more readily hydrolyzed after pepsin pretreatment, as was the case after heat treatment at pH 7.

Sugars.--The mono- and di-saccharide components of lactose were evaluated to establish their influence upon rates of proteolysis with and without application of heat. The sugars exerted effects on an individual basis not associated with chemical reactions of the sugars. Specific activities and substrate concentration optima for casein were greater in each preparation with sugar added. The proteolysis rates suggested dissociation of the micelle followed by reversible complexing as proposed by Lea and Hannan (1950). Treatment of β-lactoglobulin with sugars without heat treatment resulted in small increases in specific activity, accompanied by reduced substrate concentration optima. Heat treatment in the presence of glucose and of galactose resulted in reduction of specific activity by 50% and in doubled substrate concentration

optimum. Heat treatment of β-lactoglobulin with added lactose and with added glucose plus galactose brought about no change in rate of proteolysis.

TABLE 17.--Pepsin-pancreatin indices[a] of milk proteins and heat-treated milk proteins.

Milk Protein	Pepsin-Pancreatin Index		
	Control	Heated	Heated in Presence of Lactose
Casein	78[a] (78)[b]	68	46
α_s-Casein	80	67	70
k-Casein	64	79	76
β-Lactoglobulin	63	63	62

[a]Animal Study Indices: 78 (Rippon, 1959); 73 (Mitchell and Block, 1946).

[b]Akeson and Stahmann (1964).

SUMMARY

The milk proteins were studied during enzymatic proteolysis as model protein systems with and without heat treatment. The milk proteins used were: casein, α_s-casein, k-casein and β-lactoglobulin.

This research was divided into three parts. The first was pepsin-pancreatin proteolysis with examination of proteins during hydrolysis and assessment of amino acids and peptides freed in the digest. The second part was evaluation of milk proteins enzymatically hydrolyzed during gel filtration. And, the third part was rate of proteolysis examination by pH-stat monitoring of proton release.

Milk protein treatments included heat treatment (121 C for 30 min), sugars, chemical denaturing agents, pH adjustment and addition of calcium and sodium salts.

Enzyme released amino acid and peptide contents from unheated proteins and those heated in the presence and absence of lactose showed results necessitating the use of an amino acid index for interpretation. Amino acid indices for milk proteins reflected the amino acid release quantities and correlated with published biological data. Casein amino acids enzymatically released

were 13% lower after heat treatment than when unheated, and a 36% reduction in freed amino acids was noted after heat treatment in the presence of lactose. The enzyme liberated amino acids from α_s-casein were 16% lower in heated than unheated digests and 12% lower when heated in the presence of lactose than unheated. The k-casein amino acids enzymatically released after heat treatment were 23% lower than unheated, and heating in the presence of lactose resulted in 19% less free amino acids than when the same protein was not heated. The enzymatic release of amino acids from β-lactoglobulin was not measurably different as a result of heat treatment with or without the presence of lactose.

Six peptides were observed in trichloroacetic acid soluble portion of the digests of heat-treated protein preparations, and they were measured concurrently with amino acid analyses. The relative amounts of the peptides and amino acid quantities from protein digests indicated that two of the peptides contained predominately histidine, threonine, and leucine.

The proteins were examined by enzymatic hydrolysis during gel filtration. The milk protein with internal disulfide linkages (k-casein and β-lactoglobulin) were 24% more completely hydrolyzed during gel filtration than casein and α_s-casein, with 2 to 10 times as much intact protein remaining after proteolysis of the proteins

containing internal disulfide linkages. The proteins hydrolyzed during gel filtration were assessed for ε-amino lysine before and after proteolysis. The ε-amino lysine data were ineffective indicators of susceptibility to proteolysis during gel filtration for milk proteins after heat treatment, possibly due to interference by terminal amino groups.

The rates of enzymatic hydrolysis for milk protein preparations were assessed by pH-stat monitored proton release. The cleavage site concentration (lysine and arginine residues) was not the predominant factor influencing rate of proteolysis for milk protein systems.

The protein systems were treated with chemical denaturing agents, and the rates of proteolysis for the altered proteins were evaluated by measurement of proton release. Urea treatment of casein and β-lactoglobulin resulted in increased proteolysis rates; however upon heat treatment the rates of proteolysis were reduced which suggested formation of complexes. The treatment of milk proteins with mercaptoethanol to disrupt internal disulfide linkages resulted in slightly decreased rates of proteolysis for casein, k-casein and β-lactoglobulin; however proteolysis rates were considerably increased after heat treatment in the presence of lactose. Performic acid oxidation of milk proteins resulted in enhanced rates of proteolysis for all proteins studied, and

hydrolysis rates were not significantly influenced by heat treatment.

Calcium (0.3 M) was added to the milk protein systems which reduced the hydrolysis rate for α_s-casein by 45% and caused a fivefold increase in the β-lactoglobulin rate of proteolysis.

The milk protein systems were heated at pH 5 and pH 9 followed by assessment of enzymatic proteolysis rates by pH-stat proton release evaluation. The rates of proteolysis of casein and α_s-casein after heat treatment at pH 5 were reduced to immeasurable levels. Enzymatic hydrolysis rates were increased for k-casein (26%) and β-lactoglobulin (20%) upon heat treatment at pH 5. The rate of enzymatic proteolysis for casein reflected a 10% reduction attributable to heat treatment at pH 9, and the proteolysis rate for α_s-casein was 25% of the untreated protein.

Rates of enzymatic proteolysis of casein and β-lactoglobulin were evaluated with added glucose, galactose and lactose. The rates of proteolysis for casein were increased by glucose and galactose addition and reduced by 50% after heat treatment. β-Lactoglobulin rates of proteolysis were not extensively changed by sugar addition, with or without heat treatment.

The milk proteins studied as model protein systems reflected the individual characteristics of each protein

by behavior unique to each upon enzymatic proteolysis. The amino acid quantities released by pepsin-pancreatin digest provided the basis for an index which could be correlated with other evaluation techniques studied, proteolysis during gel filtration and hydrolysis rate measurements. The rate of protein hydrolysis assessed by proton release provides a potential method for rapid estimation of protein digestibility, as correlation was observed between rate of proteolysis and digest amino acid index.

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