IN VITRO ENZYMATIC DIGESTION OF MILK PROTEINS

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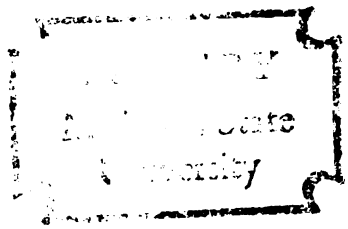
THESIS



ABSTRACT

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OF MILK PROTEINS

By

Steven R. Dimler

The groundwork was laid for an in depth study into an in vitro enzymatic approach to digesting proteins for the purpose of evaluating their nutritive value. Three types of milk proteins served as model systems: whole casein, α_s-casein, and β-lactoglobulin. A static (in flask) digest was used. End product inhibition studies indicated that amino acids do competitively inhibit the action of pancreatin on native proteins. Using relatively high concentrations of pepsin and pancreatin helped to alleviate the adverse effects of product inhibition. Expressed as mg free amino acids per 100 mg of protein digest: whole casein released 22 mg, α_s-casein released 30 mg, and β-lactoglobulin released 35 mg. Pepsin Pancreatin Digest index values for whole casein, α_s-casein and β-lactoglobulin were 78, 75, and 95 respectively.

The above proteins were subjected to prior treatment before being enzymatically digested. These consisted of autoclaving, autoclaving with equal amounts of glucose,

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and autoclaving with 5X the amount of glucose relative to protein. Only specific essential amino acid residues in casein were reduced and the total free amino acids released remained unchanged in the digests. Both α_s-casein and β-lactoglobulin digest fractions showed progressive reductions in all of the amino acids released, implying both individual and structural damage.

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INTRODUCTION

The value of new sources of proteins for human food, the effects of various processes and storage conditions, and the combination of several protein foods into the human diet to optimize their nutritive value is an area of contemporary interest. The means for analyzing the nutritive value has rested traditionally with indices such as protein efficiency ratio (PER), net protein utilization (NPU), biological value (BV), and the slope assay which are obtained from lengthy, expensive animal assays. Shorter chemical methods such as chemical score and the modified essential amino acid index (MEAA-I) often show poor correlation to animal assays. In vitro enzymatic digests offer a viable alternative to animal assays as an option for monitoring due to their low cost, quickness, and high correlation to animal assays.

The objective of this study was to further improve upon the in vitro system, identifying its strengths and weaknesses and to use the method to analyze different native and treated proteins.

REVIEW OF THE LITERATURE

Apsects of *In Vivo* and *In Vitro* Proteolysis

Peters (1970) suggested that the small peptides, three to six amino acid residues in length, released by peptic and pancreatic digestion undergo final hydrolysis to free amino acids and dipeptides by amino peptidases which, according to Matthews (1971), are secreted by the brush border or interior of mucosal cells. However, part of the digestion of small peptides occurs by the pancreatic enzymes which are bound to the brush borders in the rat (Woodley, 1969) and probably also in humans (Goldberg et al., 1971). Amino acids and dipeptides are transported into the enterocytes where dipeptide hydrolysis occurs. Holdsworth (1972) reviewed the work concerned with the absorption of dipeptides into the blood and concluded that the only known tripeptide to cross the enterocyte intact was composed of histidine, proline, and glutamic acid, a thyrotrophin releasing factor which is resistant to proteolytic enzymes. It was not clear though what proportion of an ingested protein is completely digested to amino acids before absorption.

By placing a mixture of eighteen amino acids in the jejenum of a man, Adibi et al. (1967) found that the amino acids were absorbed at varying rates. Delhumeau et al. (1962) "fed" different molar ratios of amino acids to the loop of a rat intestine and concluded that there was a higher absorption rate of a mixture simulating whole egg hydrolyzate than one simulating a casein hydrolyzate. Although the literature reports conflicting results, Itoh et al. (1973) supported observations showing higher growth rates for rats fed whole casein protein than those fed a compositionally identical amino acid counterpart. One explanation contributing to the difference in growth rates is derived from the work of Matthews et al. (1968) who noted that the transport of methionyl-methionine, methionyl-glycine, and glycyl-methionine was more rapid than the identical free amino acids.

In vivo conditions of the digestive tract include a pH range of 2 to 6 in the stomach and 4.5 to 6.5 in the small intestine (Nasset, 1957). Using meals containing up to 30 g of protein, Nixon and Mawer (1970a) estimated the secretion of endogenous protein to range from 2 to 8 g.

Digestive enzymes, following isolation and purification, exhibit definite pH values for optimum activity. For pepsin (3.4.4.1), the proteinase in the

gastic juice, Bovey and Yanari (1960) identified the optimum pH to be 1.8. The pancreatic enzymes released by the pancreas to the small intestine consist primarily of trypsin (3.4.4.4) and chymotrypsin (3.4.4.5), with optimum activities at pH 7 to 8. Smith and Hill (1960) suggested that leucine aminopeptidase (3.4.1.1), identified by its trivial name of erepsin, be given more active consideration as an important enzyme in physiologic proteolysis.

Each of the digestive enzymes mentioned above cleave the proteins differently (Hill, 1965). Pepsin, the least specific, cleaves peptide bonds in which phenylalanine, tryosine, glutamic acid, cystine and cysteine contribute either the amino or carboxyl group of the bond. Trypsin has a specificity limited to cleaving peptide bonds with lysine or arginine in the carboxyl position. Chymotrypsin cleaves the peptide bonds with tyrosine, phenylalanine and tryptophan in the carboxyl position. Leucine aminopeptidase, although showing highest hydrolysis rate with leucine residues, will cleave peptide bonds adjacent to any α-amino group. The L-isomeric form of the amino acid residue is preferred for each of the enzymes (Rupp et al., 1966).

Relationships of Various Proteins to Degree of Digestion

Proteins differing in structure and composition undergo different digestion and absorption patterns.

Epstein and Possick (1961) attributed these differences in the strengths of the stresses involved in the tertiary linkages between polypeptide chains. Allison (1964) drew an analogy stating that the degree to which a protein's structure contributes to its proteolysis is as essential and unique as behavior contributes to behavioral reactions. Zebrowski (1968) observed from studies with white rats that casein emptied from the stomach faster than did heated soya protein. The intestine also contained more soluble nitrogen from digested casein than from the heated soya diet.

The indigenous proteins of milk form a stable, colloidal suspension. Casein, comprizing 80% of the total proteinaceous material, consists of loosely packed calcium caseinate complex units joined by calcium and calcium phosphate-citrate linkages (Moor, 1967a). Thompson et al. (1965) defined casein as a heterogeneous group of phosphoproteins precipitated from skim milk at pH 4.6 and 20 C. Waugh (1961) suggested one form in which the interaction of the casein fractions stablized the micelles in casein. α_s-Casein, the major component of the casein fraction, as described by McKenzie (1967), is defined as a calcium sensitive, disordered protein without intermolecular disulfide bonds. α_s-Casein's disordered configuration allows it to undergo rapid digestion particularly by the newborn.

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β-Lactoglobulin represents 10% of the total protein content and 50% of the total whey proteins in milk. Moor (1967b) described β-lactoglobulin as a globular protein containing disulfide linkages and sulfhydryl groups susceptible to reversible disulfide interchange. Tanford et al. (1962) indicated that its native structure is compact. Refolding, following denaturation, produces a large number of α-helixes. McKenzie (1967) concluded that β-lactoglobulin exhibits changes in its secondary structure influenced by the environmental conditions.

Although normal heat processing procedures induce little nutritional damage to the protein in milk and meat products, it enhances the quality of most legume and some cereal proteins (Bender, 1972). As "new" proteins and new processing techniques gain recognition, assays capable of continuously monitoring the nutritional changes become more significant.

The structure and composition of protein species determines the effects of various potentially damaging processes. Menden and Cremer (1966) compared the effects of heat with and without added glucose on casein and on meat protein. The overall analysis indicated that in the casein fraction lysine and arginine showed the largest decline. There was some reduction in methionine and tyrosine. In meat, threonine was the only residue adversely affected.

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Extensive reviews treat the subject of protein interactions as induced by heat both in the presence and absence of a reducing sugar (Janick, 1973; Lien and Nawar, 1974a-c; and Bender, 1972). The amino acids which are generally labile to heat in the presence of reducing sugars include lysine, arginine, histidine, methionine, tryptophan, and threonine (Baldwin et al., 1951; Cook et al., 1951; Ford and Salter, 1966; Mauron, 1970; and Bender, 1972). Most studies have focused upon lysine degradation because of the prevalent Maillard condensation reaction (Henry, 1957; Holsinger et al., 1972; Carpenter, 1973; and Hurrell and Carpenter, 1974). Mauron (1970) and Carpenter (1973) observed that under mild application of heat the sugar moieties mask or block a free amino group (e.g., the ε-amino group of lysine). Baldwin et al. (1951) showed with the aid of infrared spectrophotoscropy that the amino groups were masked by masses of hydroxyl groups with the carboxyl groups unchanged in a casein-dextrose heat-treated mixture. Mauron (1970) pointed out that neither pepsin nor the pancreatic enzymes (trypsin can cleave the bond adjacent to blocked lysine amine groups. However, acid hydrolysis frees lysine of the sugar moiety, thus implying physiological availability when analyzed quantitatively. Hurrell and Carpenter (1974) stated that around 50% of the enzymatically unavailable lysine groups are recovered by acid hydrolysis. Increased levels of heating degrade

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the basic amino acids that were blocked. Proteins also undergo structural damage with the development of enzyme-resistant, intramolecular linkages between hydroxyl and amino groups. The byproducts of heat-sugar treatments, e.g., premelanoidins, may in themselves be enzyme inhibitors in the digestion processes, Adrian and Frangne (1973). Rao and Rao (1972) reported that autoclaving casein in the presence of arabinose caused the most damage, followed by glucose, then lactose. Under similar conditions, Tu and Eskin (1973) found that the following sugars caused damage in decreasing order: xylose, fructose, and glucose.

Methods of Assaying Nutritive Value of Proteins

Block and Mitchell (1946) noted that not only the total content but also the relative proportion of amino acids are essential considerations for proper growth. Since the pooling of ingested amino acids occurs only momentarily, if at all, the key to the nutritive value of a protein is the presence of an appropriate assay of amino acids present at the site of protein synthesis.

Within the framework of an *in vitro* digest, several variables exist such as the type and concentration of enzyme(s) employed, and the method of analysis. Many combinations have been employed. Sheffner *et al.* (1956) used a peptic (100:2.5, S:E) digest which was analyzed microbiologically for available amino acids to formulate

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his Pepsin Digest Ratio. After tabulating the profile of essential amino acids after consecutive digests with pepsin, trypsin, and erepsin, the greatest difference between the digests appeared following the peptic digest. Microorganisms were used to the data. However, when using microorganisms, neither a) the extent to which the various peptides contribute to the total "free" amino acids, nor b) the microorganism's specificities to consume soluble peptides rather than free amino acids are known. Baldwin (1951) indicated that microorganisms utilized a large proportion of lysine, methionine, histidine and threonine whereas none of these were utilized by the rat. Although Sheffner's values agreed closely to the biological values, Akeson and Stahmann (1964) obtained indices showing closer correlations with rat feeding trials. They used pepsin (100:1.5, S:E) followed by pancreatin (25:1, S:E) to produce a digest which was analyzed with an amino acid analyzer.

Many researchers, DeBaun and Connors (1954) and Yamashita et al. (1970), to cite two, used only a trypsin digestion as a relative gauge of lysine destruction for overall nutritive value. Valaris and Harper (1973a, b) performed inhibitory studies of the effects of carboxymethyl cellulose on α_s-casein and concluded that data obtained from a tryptic hydrolysis can be misleading if the prior peptic treatment was omitted. Menden and Cremer (1966) used relatively high concentrations of pancreatin

(2:1, S:E) and avoided using pepsin before hand to limit possible hydrolysis of the pepsin by the pancreatin. The digest ran for 15 h, a time they felt was short enough to avoid autolysis of the pancreatin enzymes. Ford and Salter (1966) reported that prolonged digestion with pepsin (10:1, S:E), pancreatin (10:1, S:E), and erepsin (3:1, S:E) released between 80-85% of the amino nitrogen as free amino acids when assayed microbiologically.

The above mentioned *in vitro* digestions were conducted in a flask under static environmental conditions. The results thus obtained are suspect when compared to the process of an *in vivo* digestion. Two aspects of the static-digest technique seem worthy of examination. First, the build-up of products of digestion may progressively inhibit further digestion. Second, the build-up of these products may induce trypsin and chymotrypsin to function as trans-peptidases. Ford and Salter (1966) constructed a Sephadex-gel filtration device to simulate a dynamic digest in which the end products were removed into the column during the course of digestion. Mauron *et al.* (1955) used a dialysis-sac digest as another means of implementing a dynamic system. He calculated a Pepsin-Pancreatin Dialysis Digest ratio index, similar to Sheffner's (1956) original index, for several proteins and found quite close agreement to the rats' biological values (Mauron, 1970). A discrepency occurred when a heat-damaged milk protein sample was assayed.

In calculating Sheffner's index, both profiles of the free amino acids in the digest and the remaining amino acids not entirely released are compared to a whole egg digest. The index shows much better correlation to rat assays than the modified essential amino acid index (Block and Mitchell, 1946), because it emphasizes the importance of having proper release and balance of essential amino acids throughout the entire digest. Supporting this concept, Melnick et al. (1946) noted that in a digest of raw soy protein, the methionine was not released until the end of the digest period. However, in the digest of heated soy, methonine was released throughout the entire course of digestion. They postulated that the disproportionate release may be a factor in lowering the nutritive value. The general conclusion drawn from the major contributors to in vitro digestion techniques supports the use of such a method as a guide toward looking at the value of native proteins as a source of human food. The index may overestimate a proteins' value, especially in treated proteins, because the total digest values have thus far been obtained by acid hydrolysis. As stated previously, acid hydrolysis releases more amino acids than appear to be released under physiological conditions.

The final criteria of the outcome of any in vitro digestion technique rests upon its correlation with biological assays. The rat has been the test animal used

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most frequently for biological assays. Protein efficiency ratio (PER) equals the weight gain divided by the amount of protein consumed, both expressed in grams. The benefits of the PER index are its ease and simplicity. However, PER cannot be used to gauge the nutritive value of all proteins for mature humans, because the amino acid requirements for a growing animal are much greater than for an adult (e.g. a growing rat has much higher lysine requirements than an adult human) whose intake of protein simply goes for maintanence and not growth. Biological value (BV) is the retained nitrogen divided by the abosrbed nitrogen. Since the BV index fails to account for digestibility differences between proteins, gross over-estimations of a protein's nutritive value can occur. Net protein utilization (NPU) is the retained nitrogen over the nitrogen intake or BV times digestibility. Although it is much more difficult to determine, NPU is overall the most accurate index for determining the nutritive value of a wide variety of proteins (Hegarty, 1975).

Inglett et al. (1969) used a computer to optimize cereal combinations where not only deficiencies but over-balances of essential amino acids were considered. Alsmeyer et al. (1974) investigated whether regression equations from a protein's amino acid composition could accurately predict the respective PER value. His equations failed to predict PER values with accuracy. Of the many foods investigated, bean foods especially high in

leucine were greatly overestimated and marine foods and noodles also gave poor estimations. Womack et al. (1974) used a rat assay to determine the effect of processing on the availability of essential amino acids. They compared an amino acid fortified casein control diet to identical diets containing a 20% reduction in one of the essential amino acids. PER values were used to follow the effects. The critical level was defined as that level of each amino acid which would maintain good PER values but when reduced 20% would yield significant drops in PER. However, their results showed that at the 20% reductions, no significant decreases in PER occurred. Abrahamsson et al. (1974) presented the "ultimate" assay for protein quality studies. They ran PER values on seven individual sources of protein as well as on many various combinations of these seven proteins. Thus, they were able to identify the most nutritive combination.

EXPERIMENTAL

Chemicals and Materials

Pepsin, hog stomach mucosa, was 1-10,000 purified. Pancreatin, hog pancreas, was 5X crystallized. Trypsin was 2X crystallized and salt free. The three enzymes were obtained from Nutritional Biochemical Corporation.

All other chemicals used in this study were obtained commercially.

Chemical Methods

Preparation of Digestion Samples for Analysis

The samples removed at various times throughout the digest were first mixed with sufficient trichloroacetic acid (TCA) to obtain a final concentration of 15% TCA. After setting overnight at 4°C, the samples were centrifuged at 1000X g for 5 min to remove precipitated material. The supernatant was clear and used for further analysis of its nitrogen, alpha amino nitrogen and free amino acids.

Nitrogen

A micro-Kjeldahl apparatus was used to determine nitrogen in both protein and deproteinated samples (Mangino, 1973). The digestion mixture contained 5.0 g $CuSO_4 \cdot 5H_2O$ and 5.0 g SeO_2 in 500 ml concentrated H_2SO_4.

Either 15 mg of dried protein or an equivalent concentration of the TCA soluble peptides were digested in duplicate by adding 4 ml of the digestion mixture and boiling for 1 h. After the flasks cooled, 1 ml of 30% H_2O_2 was added and the digestion continued by boiling another hour. After cooling the flasks, the sides were rinsed with 10 ml of deionized water. Neutralization was accomplished by adding 25 ml of a 40% NaOH solution. The free ammonia was steam distilled into 15 ml of a 4% boric acid solution containing 5 d of indicator consisting of 400 mg bromcresol green and 40 mg of methyl red dissolved in 100 ml of 95% ethanol. The termination of distillation occurred when the receiving beaker reached a volume of 60 ml. The ammonium borate complex was titrated with 0.0230 N HCl previously standardized by tris-hydroxymethylaminomethane as a primary standard.

$$\%N = \frac{(\text{ml sample} - \text{ml blank})(N)(14.007)(100)}{\text{mg sample}}$$

Alpha Amino Nitrogen -- Ninhydrin Test

Appropriate aliquot volumes of the deproteinated samples were pipetted into test tubes for further dilution. The dilution factors required ranged from 5, 50, to 100 for the samples from the enzyme blanks, the peptic digest, and the second pancreatic digest respectively. When the proper dilution was obtained the samples were assayed by the ninhydrin test (Clark, 1964). One-half milliliter of the sample was mixed with 1.5 ml of the ninhydrin reagent

solution[a], then set in a boiling water bath for 20 min while topped with glass marbles to prevent evaporation losses. Following the heat treatment, the reaction mixtures were cooled to room temperature followed by the addition of 8.0 ml of 50% n-propanol. Color development stabilized after 10 min. Absorbance readings were recorded at 570 nm wavelength using a Beckman DK-2, double-beam spectrophotometer. Glycine was used to prepare a standard curve.

Free Amino Acids

The samples analyzed were deproteinated by TCA and corresponded to a peptic-pancreatic digestion of a total of 26.5 h. The precipitated proteins and large peptides were centrifuged and filtered through a 0.22 μm Millipore filter apparatus, yielding a crystal clear, final solution. An aliquot of this solution was mixed with a standard concentration of nor-leucine which served as an internal standard. The particular nor-leucine solution reacted with the salts in the TCA solution, producing a cloudy appearance. An additional filtering using the Millipore apparatus removed the cloudiness. The samples were then ready for analysis in a Beckman/Spinco Model 121 C Amino Acid Analyzer according to the procedure of Spackman et al. (1958), Moore and Stein (1954) and Moore et al. (1958)

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[a]See Appendix for the list of ninhydrin reagents.

Physical Methods

Preparation of Whole Casein

Whole casein was precipitated from fresh skim milk at 38 C by the addition of 4N NCl to pH 4.6. The precipitated protein was collected with double layered cheesecloth and squeezed dry. The casein was redissolved in distilled water using 1 N NaOH to maintain a constant pH of 7.5. The procedure was repeated until a caseinate fraction washed and reprecipitated a total of four times was obtained. After the final dissolution, lyophilization was begun and the dried protein stored at -20 C. Henceforth, the sodium caseinate fraction will be referred to as whole casein.

Preparation of α_s-Casein

The method used closely followed that of El-Negoumy (1966). Approximately 350 g of frozen whole casein was dissolved in 1 l of a 6.6 M urea solution. An addition of 200 ml of 7 N H_2SO_4 lowered the pH to 1.4. Following the addition of 2 l of distilled water, which diluted the urea to 2.2 M, an α_s-casein-rich fraction precipitated. The centrifuged precipitate underwent purification steps by redispersion in 1 l of a 6.6 M urea solution. Diluting with distilled water to attain a 4.8 M urea concentration caused some precipitation of residual α_s-casein, however most of the further enriched fraction was precipitated at a 3.3 M urea concentration. After redissolving in a

4.8 M urea plus 21.8 g NaCl per 1, an electrophoretically pure fraction was obtained upon dilution with distilled water to both 3.3 M and 1.7 M urea. The precipitate was washed several times with distilled water, then redissolved in water maintained at pH 7.5 with NaOH, dialyzed for 36 h against distilled water at 4 C, lyophilized, and stored at -20 C as sodium α_s-caseinate.

Preparation of β-Lactoglobulin

The method used followed that suggested by Fox et al. (1967). The whey portion from the first precipitation of whole casein was carefully decanted into another beaker. Enough trichloroacetic acid (TCA) was added to the acid whey to give a final concentration of 3% TCA. After standing for 30 min, the precipitate was removed by centrifugation and discarded. The clear supernatant was poured into cellulosic, dialysis sacs and pervaporated to one-tenth its original volume. Ammonium sulfate was added to yield a 0.4 saturated solution. The residual precipitate was discarded. Ammonium sulfate was added to saturation. After standing overnight at 4 C, the precipitated fraction was collected by centrifugation and purified by a second suspension in a saturated ammonium sulfate solution. The precipitate was dissolved in 400 ml of distilled water with 1 N NaOH to keep a constant pH of 7.5, placed in a dialysis sac and dialyzed against distilled water at 4 C. The solution was lyophilized and stored at -20 C.

Acrylamide Gel Electrophoresis

Homogeneity of the milk protein fractions was assessed electrophoretically by a modified version of the method by Melachouris (1969). This procedure called for two gel systems, but only a running gel and spacer buffer were used. The running gel was prepared by dissolving 45 g of Cyanogum-41 in 0.380 M tris-HCl buffer at pH 8.9 then making up to 500 ml. A spacer buffer consisted of a 0.062 M tris-HCl pH 6.7 buffer. These solutions were used for β-lactoglobulin. The same solutions containing 5 M urea were used to assay the casein fractions. The stock, electrode-vessel buffer contained 0.046 M tris-glycine at pH 8.3.

Protein samples were dissolved in the spacer buffer, which served to "stack" the proteins during electrophoresis. Several sucrose crystals and bromophenol blue provided the necessary density and marker dye, respectively. To 20 ml of the running gel, 0.02 ml of N,N,N',N'-tetramethylethylenediamine was added followed by 0.07 ml of 5% ammonium persulfate. A small amount of water was layered over the top of the gels to insure a level surface. The gel set in 20 min. During the run, a constant instrument voltage of 150v was applied. The gels were removed, stained with amido black, and destained in a methanol-acetic acid solution.

Treatment of Milk Proteins

The milk proteins were analyzed under four different treatments: a) native, untreated, b) autoclaved for 30 min at 15 psi, c) autoclaved with equal amounts of glucose, and d) autoclaved with 5X the amount of glucose.

The protein samples were prepared in concentrations of 12 mg of protein sample per ml water at pH 7.0. The solutions were poured into 125 ml flasks and covered with aluminum foil. Heat treatments were conducted in a Masco Cyclomatic Sterilizer. Evaporation losses were corrected after cooling. The samples were stored at 4 C.

Enzymatic Digestion

The pH of untreated and treated protein samples was lowered to 1.8 with 1 N HCl. The volume was increased from 25 to 30 ml, representing a sample concentration of 1.0%. Twenty-five mg of pepsin was dissolved in the solution. After swirling, the flask was placed in a 37 C oven for 20 h of incubation.

Peptic digestion was terminated by the addition of a 0.5 M $NaHCO_3$ buffer (pH8) and 1 N NaOH until a stable pH of 8 was obtained. A final increment addition of distilled water brought the total volume added to 5.0 ml. Thirty mg of pancreatin was dissolved into the reaction mixture. Reincubation at 37 C lasted for 3 h followed by a second addition of 30 mg of pancreatin for an additional 3.5 h incubation.

Enzyme blanks were treated identically except for the absence of the specimen protein.

pH-Stat Evaluations

A sargent pH-Stat was calibrated to maintain each sample at 37 C at pH 8.0. Standard volumes used were 10 ml of substrate solution and 0.2 ml of enzyme solution. A magnetic stirring bar maintained continuous mixing. The addition of the proton-neutralizing 0.05 N NaOH solution was monitored on the recorder chart.

A slight buffering action helped to insure stable monitoring by the pH-Stat. The buffer selected contained 0.605 g of TRIS and 2.34 g of NaCl/l plus a concentration Of 0.02% Na azide to inhibit microbial growth. A stock protein solution of 1% whole casein dissolved in the buffer was prepared. The stock protein solution was diluted to vary substrate concentrations. Initially, 0.05 M $CaCl_2$ was used to stabilize the trypsin solution against autolysis. However, the Ca (II) caused the casein to precipitate, thus inducing an extraneous drop in pH. Keeping the enzyme solution in an ice bath retained its stability and using 0.20 ml solution per reaction did not affect the stabilized temperature in the reaction vessel. A mixture of nine of the water soluble amino acids: glycine, arginine, serine, alanine, histidine, lysine, valine, phenylalanine and methionine at a total concentration of 48 mg/ml, comprized one inhibitor solution. Other inhibitor solutions included a 1% casein

solution digested to completion, as monitored by the pH-Stat, with either trypsin or pancreatin.

The substrate was allowed to achieve pH and temperature stability before the enzyme was injected into the reaction vessel. The injection caused no change in the two parameters. The pH-Stat measured the extent of enzymatic hydrolysis of peptide bonds by maintaining a constant pH through the continuous addition of base. An attached chart recorded the amount of base released over time. Initial velocity rates were measured from the slope of the reaction curve with the aid of a mirror as described by Bergmeyer (1963).

The pH-Stat had a maximum release of base over time of 6 units base/unit time. It was found that the initial rate of digestion of 0.010 g/ml of substrate by 2 mg of trypsin was at the maximum value of 6. Hence, the pH-Stat could not record any higher initial reaction rates with digestions containing higher substrate concentrations.

The method used in this study to analyze the data from the reaction rate study was by plotting reciprocal velocity vs reciprocal substrate concentration (Lineweaver-Burke plot). By definition, the y-coordinate would represent the reciprocal maximum initial velocity of the reaction. Thus during the tryptic digestion in this study, the $1/V_{max}$ value of .17 corresponded to a 1/(S) value of 100. For the purpose of clarity, the graph was

plotted using a 1/(S) value of 100 at the y-coordinate position. However, in order to calculate a linear regression analysis on the plotted points, the 1/(S) values (x-axis) had to be all subtracted by 100. The data from pancreatic digestion study was handled similarly, where 1/(S) assumed the value of 68 at a $1/V_{max}$ of .17.

The data was also plotted on an Augustinsson plot ((S)/v vs (S)). Again, the actual substrate concentration value used had to be adjusted to reflect the physical limitation of the pH-Stat's monitoring capacity. The method used to adjust the values included 1) subtracting the 1/(S) value corresponding to the $1/V_{max}$ value of .17 from all of the 1/(S) values in order to obtain "adjusted" 1/(S) values, 2) inverting them to obtain "adjusted" (S) values, and then 3) using the "adjusted" (S) values to plot the graph of the (S)/v vs (S). Identical slopes between the control digestion and those with inhibitors would imply identical V_{max} values. Although not submitted into this thesis, Augustinsson plots were drawn and agreed with the conclusions of the Lineweaver-Burke plots.

RESULTS

Product Inhibition

Trypsin

The purpose of the experiments with the pH-Stat was to explore any evidence of product inhibition on the activity of trypsin and pancreatin. The inhibitors employed were selected in an attempt to simulate varying degrees of proteolysis, ranging from large peptides to free amino acids. They included 1) a tryptic digest, 2) a peptic-tryptic digest, and 3) a mixture of lysine and arginine. The data obtained are tabulated (Table 1) for application to a Lineweaver-Burke plot interpretation. A 0.15% concentration of the inhibitor yielded interpretable results. Higher concentrations of the pre-digested fraction produced greater inhibition, to the point where virtually no enzymatic hydrolysis was detected in a 0.50% protein plus 0.50% pre-digested fraction. Data in Figure 1 illustrates the effect of the inhibitors. As indicated, the V_{max} of each "digestion" remained constant, thus implying a condition representative of competitive inhibition.

Pancreatin

The data collected from the pancreatin study is listed in Table 1. The inhibitors employed consisted of different concentrations of a mixture of nine, water soluble amino acids[a]. The ratio of whole, intact protein to free amino acids was 2:1 and 1:1. Figure 1 indicates a uniform V_{max} implying the inhibition as a competitive type. The latter reaction ratio produced an initial velocity half that of the control rate when using a 0.50% substrate concentration.

Various observations in this study merit attention. After increasing the concentration of pancreatin to 10 mg/0.20 ml, the initial velocity was one-half that of trypsin at 2 mg/0.20 ml. However, the total amount of protons released over a longer time span was more than in the tryptic digest. Pancreatic digest of a pepsin-digested substrate produced lower reaction rates than in an intact substrate sample. The peptic digested sample would contain a smaller number of peptide bonds and the increase in free amino acids contribute to enzyme inhibition.

Digests with Varying Amounts of Enzyme

The purpose in varying enzyme concentration was to obtain maximum digestion with minimum enzyme contribution.

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[a]See Appendix for the contents of each specific amino acid.

Figure 2 illustrates the relative extent of digestion arising from varying enzyme concentrations. A Uniform 1% whole casein solution served as the substrate. The peptic and pancreatic digestions were for 3 h and 6.3 h, respectively. The rate of digestion was monitored by the ninhydrin test on TCA-soluble supernatants. Four different digestions were conducted, each with different enzyme concentrations regarding pepsin and pancreatin. The digests were initiated with 4.5 mg, 10 mg, 10 mg, and 25 mg of pepsin. After 3 h, the amount of pancreatin added to the aforementioned digestions was 12 mg, 12 mg, 30 mg, and 30 mg, respectively. Three hundred mg of whole casein was present. The flask with the lowest enzyme concentration typlified that of the digestion procedure of Akeson and Stahmann (1964).

As illustrated in Table 2, the pepsin blanks showed a minimum contribution to the ninhydrin positive reaction of the system. The pancreatin blanks induced a greater response. However, in both cases, the increase in absorbance, or the degree of cleavage, was slight and uniform over the time observed.

Figure 2 shows the patterns of the protein digests with the enzyme blank values subtracted. The final points indicate that the degree of digestion achieved with the higher concentration of enzyme was at least twice that of the digestion obtained with the lowest enzyme concentration.

Milk Protein Fractions

Amino Acid Profile

The amino acid composition of each of the three proteins studied was obtained after acid hydrolysis in evacuated, sealed ampules for 22 h at 110 C. Compositions were compared to published values as reported in Table 3. Examination of the results indicates close agreements. Deviations may stem from differences in preparative techniques, purity of specimens, analytical methods and genetic variations.

Color Changes During Treatments

Prior to the heat treatment, all of the protein samples were white in color. Neither whole casein nor α_s-casein solutions showed indications of browning after autoclaving. However, autoclaving turned the β-lactoglobulin solution slightly tan in color. Heating the proteins with 1% glucose altered the appearances as follows: whole casein became amber-brown, α_s-casein turned tan, and β-lactoglobulin was tannish yellow. By increasing the glucose concentration to 5%, heating produced a reddish-brown whole casein, a brownish-tan α_s-casein, and a yellowish-orange β-lactoglobulin solution.

Effects of Treatments on the Rate of Digestion

Table 4 lists the data for all of the digestion experiments monitored by the ninhydrin test. The

preceeding Figures 3, 4 and 5 illustrate the relative degree of digestion with time. Preliminary experiments in this study revealed that the reduced degree of digestion in a 3 h compared to 20 h peptic digest was overcome by the pancreatic digest. Kjeldahl analysis indicated that after 20 h pepsin and 6.5 h pancreatin (twice added), the TCA-soluble peptides represented 95% or more of the total amount of protein present.

Enzymatic Digestion of Protein Samples

Amino Acid Profile

The amino acids liberated by the pepsin-pancreatin sequential proteolysis of the milk protein samples are listed in Tables 5, 6, and 7. Each table shows a comparison of the amino acid profile enzymatically released as amino acids per ml of digest, corrected for the enzyme blank, with the theoretical concentration based on the amount of protein recovered by acid hydrolysis. A high voltage electrophoretic spot test supported the evidence that few large peptides were present in the final digest. As previously mentioned, Kjeldahl analysis showed that at least 95% of the digest was soluble in TCA.

Whole Casein

Data for the digestions of the whole casein samples are presented in Table 5. Due to destruction by acid hydrolysis of the native protein, values for ½ cystine

and tryptophan represent estimations obtained from Block and Weiss (1956). As expected, according to the specificities of the enzymes used, over 40% of arginine, tyrosine, leucine, phenylalanine, lysine, tryptophan, and methionine were released from the untreated substrate. Of the essential amino acids, isoleucine and valine ranked the lowest in amount, accounting for only 10.0% and 9.1%, respectively, of the theoretical total. Low yields of free aspartic acid, glutamic acid, glycine, and proline were noted.

Table 8 lists the essential amino acids showing a reduction in amount released compared to the digestion of untreated protein. Autoclaving caused a reduction of 7% for lysine, 22% for arginine, and 5% for methionine. Including 1% glucose into the heat treatment caused further reductions; 12% for lysine, 22% for histidine, 46% for arginine, 7% for methionine, and 28% for tryptophan. Increasing the glucose concentration to 5% induced decreases of 48% for lysine, 45% for histidine, and 24% for methionine. Arginine and tryptophan showed slight increases in relation to the percent decrease following the heat plus 1% glucose treatment.

As the glucose concentration was increased further decreases in the total amount of amino acids enzymatically released were slight. The total free amino acids released was approximately 22 mg/100 mg protein. Since the total

amount was practically unchanged, selected amino acids showed increases compared to the digest of treated whole casein. The residues involved represent the non-polar, uncharged amino acids: valine, isoleucine, leucine, and phenylalanine.

α_s-Casein

Table 6 lists the results for the digestions of the α_s-casein samples. Due to the destruction of tryptophan by acid hydrolysis of the native protein, its value was estimated from Dayhoff (1972). The release of individual, essential amino acids in the digest of the untreated sample expressed as a percentage of the total amount present in the intact protein ranged from 91% for lysine, 70% to 80% for methionine, arginine and tryptophan, 60% for phenylalanine, 45% for leucine, 33% for histidine, and less than 15% for valine and isoleucine. Overall, the digest of the untreated α_s-casein fraction contained 30% by weight of free amino acids.

Table 8 lists the essential amino acids reflecting a reduction in amount released compared to the digestion of the untreated protein. Autoclaving alone resulted in reductions of 29% for valine, 14% to 19% for methionine, isoleucine, and lysine, and 8% to 9% for phenylalanine and arginine. Autoclaving with 1% glucose caused greater reductions of 38% to 45% for lysine, valine, isoleucine, methionine, and arginine, 22% for leucine, 16% for phenylalanine, and 7% for histidine. Increasing the glucose

to 5% in the autoclaved sample induced little additional change in the amounts released for valine, methionine, isoleucine, leucine, and phenylalanine. However, lysine, arginine, histidine, and tryptophan dropped to 61%, 55%, 23%, and 19%, respectively. The amount of free amino acids released per 100 mg of protein dropped from 30% for the untreated, 27.5% for the autoclaved, 23% for the autoclaved plus 1% glucose, and 21% for the autoclaved plus 5% glucose.

β-Lactoglobulin

Table 7 lists the results for the digestions of the β-lactoglobulin samples. Due to the destruction of tryptophan and partial destruction of methionine by acid hydrolysis, their values represent estimations from Dayhoff (1972). The release of individual, essential amino acids in the digest of the untreated sample expressed as a percentage of the total amount present in the protein ranged from 70% for arginine, 55% to 60% for lysine, methionine, leucine and tryptophan, 39% to 43% for isoleucine, valine, and phenylalanine, and 31% for histidine. Overall, the free amino acids in the total digest was 34.6%.

Table 8 lists the essential amino acids reflecting a reduction in amount released compared to the digest of the untreated protein. Autoclaving caused reductions from 24% to 29% for lysine, methionine, tryptophan and

leucine. The remaining values represent from 10% to 20% reductions. The effect of 1% glucose plus heat caused decreases of 47% for methionine, 44% for arginine, 36% to 39% for lysine, leucine and phenylalanine, 28% to 30% for valine, isoleucine and tryptophan, and 23% for histidine. Only lysine, tryptophan and arginine decreased further with the treatment of heat plus 5% glucose, they were reduced to 48%, 42%, and 55%, respectively. The amount of free amino acids released per 100 mg of protein dropped from 34.6% for the untreated, 28% for the autoclaved, 22.5% for the autoclaved plus 1% glucose, and 22% for the autoclaved plus 5% glucose.

Pepsin Pancreatin Digest Index

Pepsin Pancreatin Digest (PPD) index were determined for each of the untreated, milk proteins by using the method of Akeson and Stahmann (1964) which was a modification of Sheffner's et al. (1955) original method. The values for the whole egg digest were obtained by digesting dried whole egg solids exactly as with the milk proteins. A 22 h acid hydrolysis produced a composition of amino acids considered to represent the total amount. The milk proteins gave PPD index values of 78 for casein, 75 for α_s-casein, and 95 for β-lactoglobulin. Whole egg[a] was estimated to be 96.5 (Akeson Stahmann, 1965).

[a]See Appendix for the amino acid composition of the enzymatic digest and the acid hydrolyzate of whole egg.

Table 1. Effects of end products on the proteolytic action of trypsin and pancreatin on whole casein

Trypsin[a]				Pancreatin[e]			
(S)[b]	1/(S)	1/v[c]	(I)[d]	(S)[b]	1/(S)	1/v[c]	(I)[d]
.0100	100	.17	-	.0100	100	.25	-
.0067	150	.18	-	.0070	143	.37	-
.0050	200	.19	-	.0050	200	.48	-
.0040	250	.21	-	.0085	118	.28	.0024[z]
.0030	330	.24	-	.0070	143	.40	.0024[z]
.0085	118	.21	.0015[w]	.0050	200	.50	.0024[z]
.0070	143	.26	.0015[w]	.0085	118	.31	.0048[z]
.0050	200	.35	.0015[w]	.0070	143	.45	.0048[z]
.0085	118	.20	.0015[x]	.0050	200	.73	.0048[z]
.0070	143	.25	.0015[x]				
.0050	200	.30	.0015[x]				
.0070	143	.26	.0020[y]				
.0050	200	.34	.0020[y]				

[a]Used 2 mg for each digest.

[b]Substrate concentration expressed as g/ml.

[c]Reciprical initial velocity rates, average of four measurements, expressed as units base/unit time.

[d]Inhibitor concentrations, expressed as g/ml, for (w) tryptic digest, (x) peptic-tryptic digest, (y) equal mixture of lysine and arginine, and (z) mixture of nine amino acids.

[e]Used 10 mg for each digest.

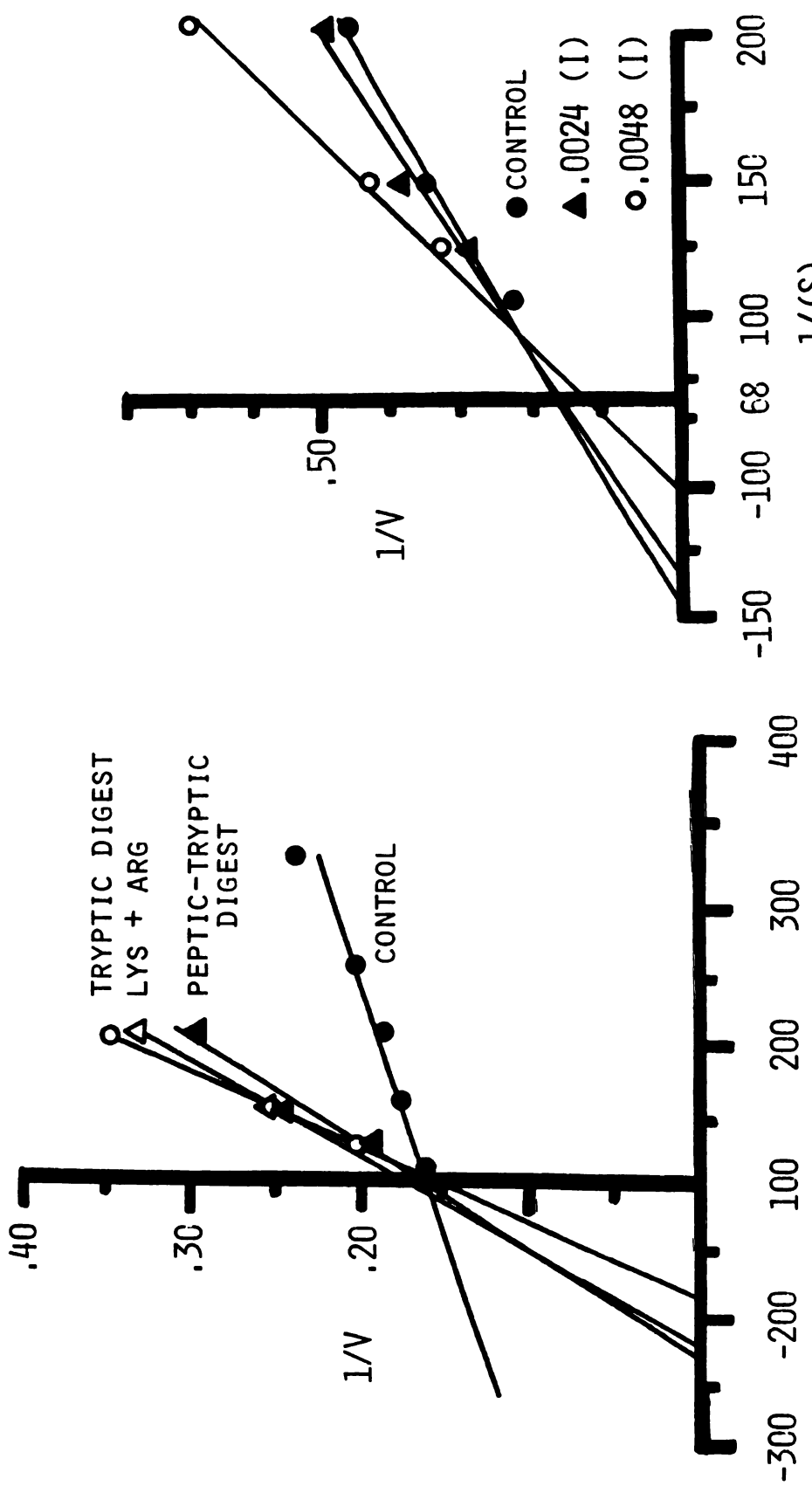

Figure 1. Lineweaver-Burke plot illustrating effects of end product inhibition on tryptic (left) and pancreatic (right) digest of whole casein.

Table 2. The effects of varying amounts of pepsin and pancreatin upon the rate of digestion of whole casein

Digest time (h)	Substrate - enzyme blank[a]				Enzyme blank[a]			
	I[b]	II[c]	III[c]	IV[e]	E-I[b]	E-II[c]	E-III[d]	E-IV[e]
pepsin								
0.1	2.64	6.32	3.49	11.73	0.13	0.28	0.21	0.36
3.0	4.92	9.21	7.31	14.80	0.18	0.29	0.34	0.46
pancreatin								
3.1	-	-	-	-	1.56	1.61	2.80	2.67
5.1	6.94	15.20	24.20	30.72	-	-	-	-
9.3	14.10	17.80	28.60	29.84	2.00	2.30	3.20	3.26

[a]Expressed as total absorbance units (see ninhydrin test) for each TCA soluble digest sample.

[b]Group I corresponds to 60:1 and 25:1 (S:E) ratios for pepsin and pancreatin.

[c]Group II corresponds to 30:1 and 25:1 (S:E) ratios for pepsin and pancreatin.

[d]Group III corresponds to 30:1 and 10:1 (S:E) ratios for pepsin and pancreatin.

[e]Group IV corresponds to 12:1 and 10:1 (S:E) ratios for pepsin and pancreatin.

Table 2. The effects of varying amounts of pepsin and pancreatin upon the rate of digestion of whole casein

Digest time (h)	Substrate - enzyme blank[a]				Enzyme blank[a]			
	I[b]	II[c]	III[c]	IV[e]	E-I[b]	E-II[c]	E-III[d]	E-IV[e]
pepsin								
0.1	2.64	6.32	3.49	11.73	0.13	0.28	0.21	0.36
3.0	4.92	9.21	7.31	14.80	0.18	0.29	0.34	0.46
pancreatin								
3.1	-	-	-	-	1.56	1.61	2.80	2.67
5.1	6.94	15.20	24.20	30.72	-	-	-	-
9.3	14.10	17.80	28.60	29.84	2.00	2.30	3.20	3.26

[a]Expressed as total absorbance units (see ninhydrin test) for each TCA soluble digest sample.

[b]Group I corresponds to 60:1 and 25:1 (S:E) ratios for pepsin and pancreatin.

[c]Group II corresponds to 30:1 and 25:1 (S:E) ratios for pepsin and pancreatin.

[d]Group III corresponds to 30:1 and 10:1 (S:E) ratios for pepsin and pancreatin.

[e]Group IV corresponds to 12:1 and 10:1 (S:E) ratios for pepsin and pancreatin.

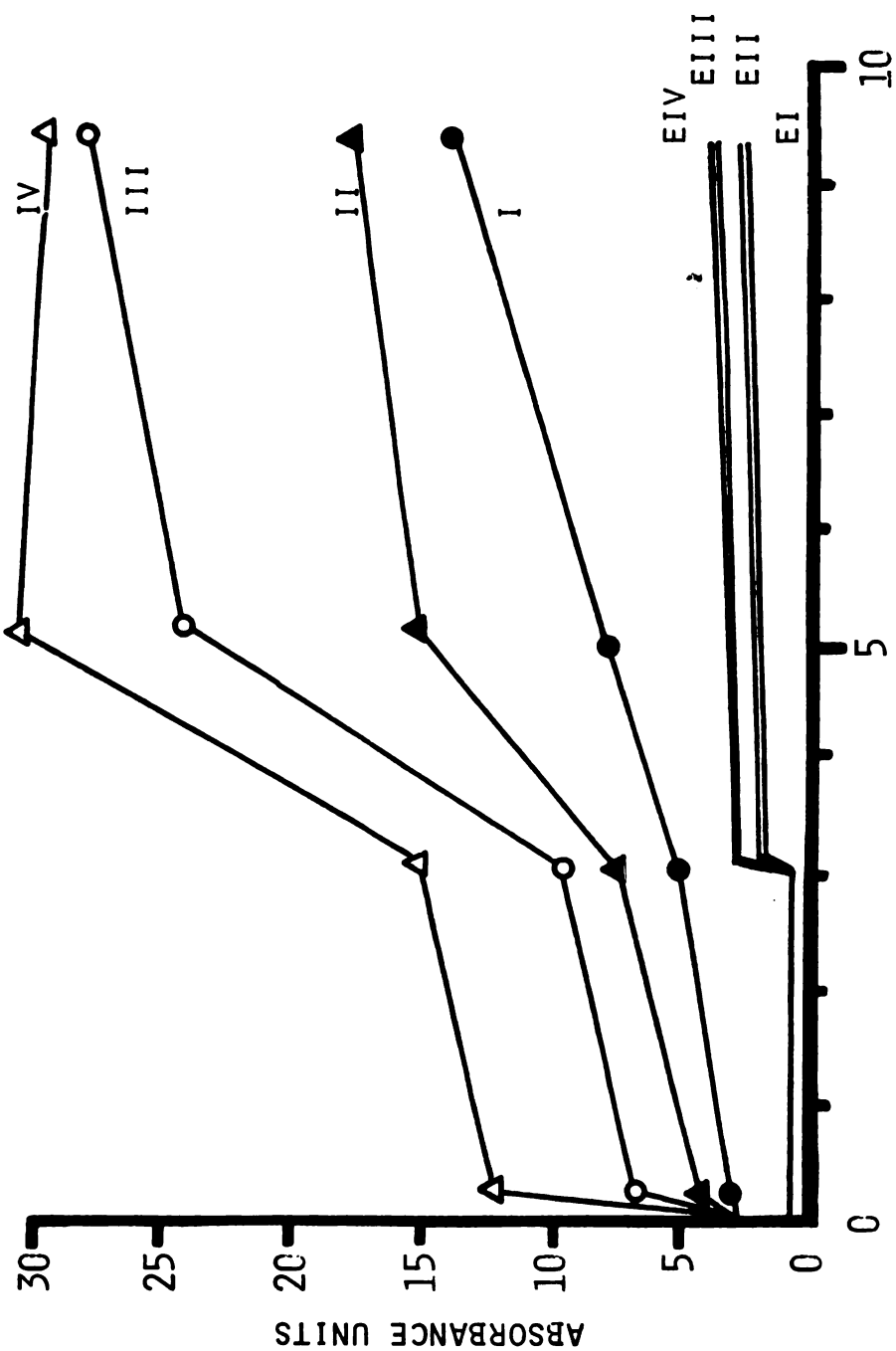

Figure 2. Rates of digestion of casein with varying amounts of pepsin and pancreatin

Table 3. Amino acid composition of proteins studied compared to published values (expressed as g/16g N)

Amino acid residue	Whole Casein		α_s-Casein		β-Lactoglobulin	
	This Study	(1)	This Study	(2)	This Study	(2)
Lys	7.8	7.1	4.4	8.9	9.4	11.0
His	3.3	2.6	2.6	3.4	2.7	1.6
Arg	3.8	3.7	3.8	4.6	2.3	2.7
Asp	6.2	5.6	8.2	4.0	10.3	9.9
Thr	3.8	3.8	2.3	2.5	4.6	4.6
Ser	3.9	5.2	5.3	6.9	3.8	3.5
Glu	21.2	20.2	11.4	15.8	16.0	14.0
Pro	10.0	10.3	8.1	8.2	4.4	4.4
Gly	1.4	1.6	3.1	2.5	0.9	1.0
Ala	2.5	2.4	2.4	3.2	5.5	5.7
1/2 Cys	-	0.3	-	-	1.8	2.9
Val	6.8	6.3	7.4	5.4	5.8	5.7
Met	2.4	2.9	2.1	3.2	3.0*	3.0
Ile	5.0	5.7	6.0	6.2	5.7	6.5
Leu	9.0	8.7	12.3	9.5	13.9	14.3
Tyr	5.4	5.7	10.4	8.1	3.9	3.7
Phe	5.0	5.2	7.3	5.8	3.8	3.4
Try	2.5*	2.5	2.5*	1.8	2.1*	2.1
TOTAL N		15.4		14.1		15.2

(1) Block and Weiss (1956) (2) Dayhoff (1972).

*Value was estimated.

Table 4. Rate of pepsin-pancreatin digest of milk proteins with and without treatments[a]

Digest time (h)	Whole casein				α_s-Casein				β -Lactoglobulin			
	Control	Heat	Heat + 1% Glucose	Heat + 5% Glucose	Control	Heat	Heat + 1% Glucose	Heat + 5% Glucose	Control	Heat	Heat + 1% Glucose	Heat + 5% Glucose
Pepsin												
0.0	1.40	1.46	1.38	1.36	1.55	1.60	1.65	1.70	1.63	1.75	1.70	1.60
4.4	7.66	7.44	6.60	6.14	11.13	11.38	11.55	11.72	6.60	6.20	5.60	5.64
20.0	9.65	10.41	10.24	9.02	13.56	14.41	14.86	14.86	14.96	14.88	13.26	12.42
Pancreatin												
20.7	20.70	19.90	18.95	16.90	22.00	21.00	22.05	22.00	23.80	21.50	20.80	0.90
23.0	22.70	23.70	22.30	21.75	24.45	24.95	23.70	22.80	30.90	25.50	26.86	26.90
Pancreatin												
24.1	22.95	23.70	21.80	20.40	25.42	24.96	24.22	23.00	31.20	26.32	28.00	27.94
26.5	25.00	25.30	24.00	22.00	26.40	25.40	24.85	23.80	34.20	28.30	29.20	29.30
44.0	29.70	30.30	28.30	26.20	30.80	29.50	28.44	27.26	43.80	38.40	35.40	34.80

[a]Enzyme blank was subtracted from the TCA supernatant digest values expressed as total absorbance units (see ninhydrin test) for 1% protein samples.

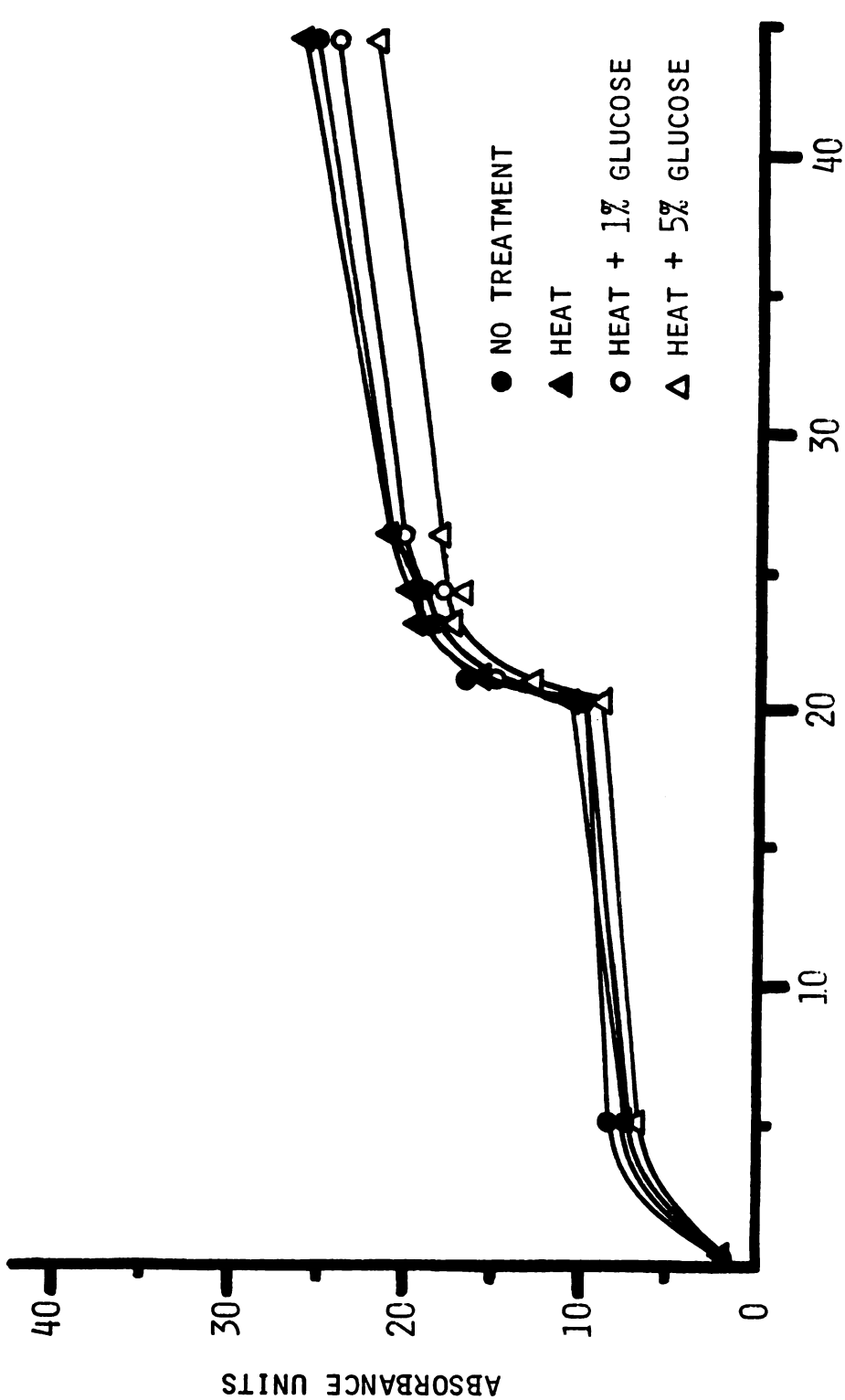

Figure 3. Rate of digestion of whole casein by pepsin-pancreatin-pancreatin

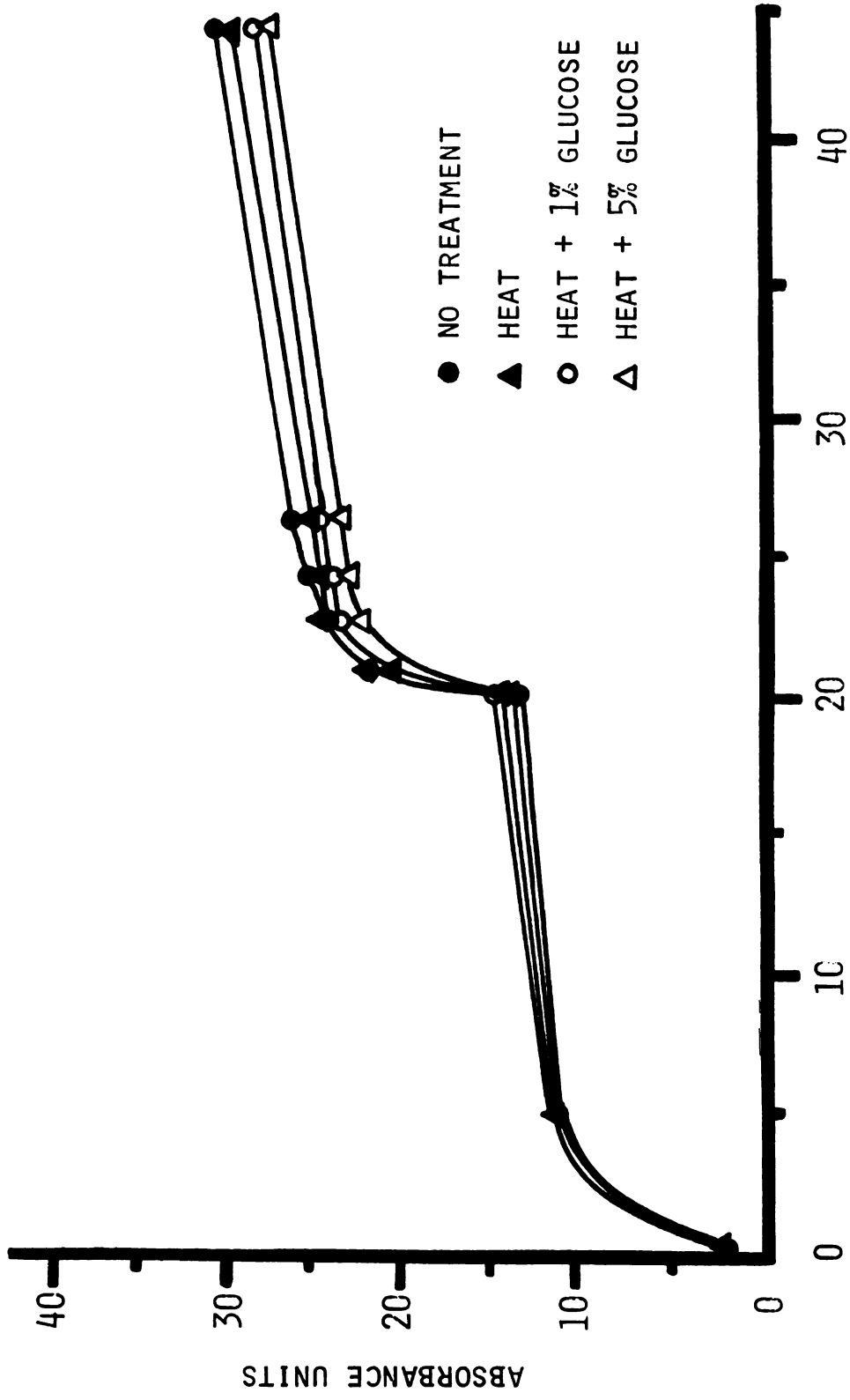

Figure 4. Rate of digestion of α_s-casein by pepsin-pancreatin-pancreatin

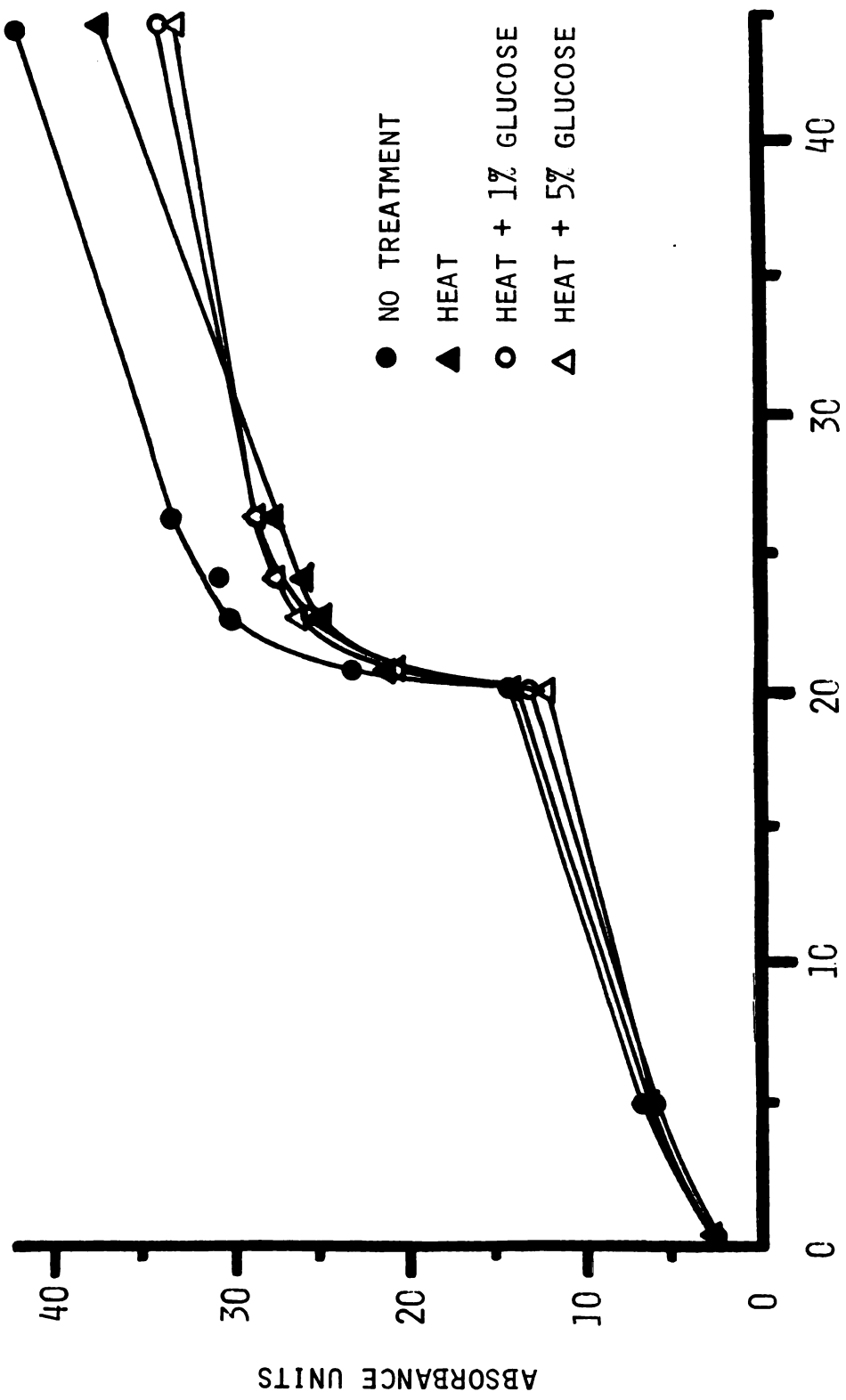

Figure 5. Rate of digestion of β-lactoglobulin by pepsin-pancreatin-pancreatin

Table 5. Free amino acids in pepsin-pancreatin digest of whole casein samples

Amino acid residue	Theoretical total[a]	Control		Heated		Heat + 1% glucose		Heat + 5% glucose	
		Digest[b]	(%)[c]	Digest[b]	(%)[c]	Digest[b]	(%)[c]	Digest[b]	(%)[c]
Lys	7.77	3.36	43.2	3.13	40.3	2.94	37.8	1.74	22.4
His	3.28	0.77	23.4	0.83	25.4	0.60	18.3	0.42	12.9
Arg	3.77	2.03	53.9	1.59	42.2	1.10	29.1	1.27	33.8
Asp	6.20	0.27	4.3	0.33	5.3	0.44	7.1	0.56	9.0
Thr-Ser	7.70	1.56	20.5	1.64	21.3	1.75	22.7	1.14	14.9
Glu	21.22	0.50	2.4	0.66	3.1	0.67	3.2	1.41	6.7
Pro	10.01	-	-	-	-	-	-	-	-
Gly	1.44	0.01	1.0	0.05	3.3	0.06	4.0	0.03	2.4
Ala	2.48	0.32	13.1	0.42	16.8	0.42	16.8	0.39	15.8
½ Cys	-	-	-	-	-	-	-	-	-
Val	6.81	0.62	9.1	0.72	10.5	0.92	13.5	0.87	12.7

Met	2.41	1.48	63.4	1.41	58.3	1.40	58.0	1.13	47.0
Ile	5.04	0.51	10.0	0.54	10.7	0.56	11.2	0.66	13.1
Leu	9.02	4.05	44.9	4.27	47.3	4.49	48.7	4.44	49.2
Tyr	5.41	2.67	49.3	2.96	54.6	3.04	56.2	3.27	60.4
Phe	5.00	2.19	43.8	2.50	50.0	2.52	50.5	2.90	58.1
Try	2.50	1.26	50.1	1.30	51.9	0.91	36.5	1.06	42.4
TOTAL	100.06	21.60		22.35		21.82		21.29	

[a]Acid hydrolyzed native protein in 6N HCl for 22 h, values expressed as g/100 g digest.

[b]Values expressed as g/100 g digest.

[c]Values expressed as per cent of the theoretical total.

Table 6. Free amino acids in pepsin-pancreatin digest of α_s-casein samples

Amino acid residue	Theoretical total[a]	Control		Heated		Heat + 1% glucose		Heat + 5% glucose	
		Digest[b]	(%)[c]	Digest[b]	(%)[c]	Digest[b]	(%)[c]	Digest	(%)[c]
Lys	4.43	4.03	91.0	3.25	73.4	2.49	56.2	1.57	35.4
His	2.62	0.86	32.8	0.90	34.4	0.80	30.5	0.66	25.2
Arg	3.81	2.93	76.9	2.67	70.1	1.63	42.8	1.31	34.4
Asp	8.23	0.35	4.2	0.26	3.2	0.57	6.9	0.60	7.3
Thr-Ser	7.56	0.95	12.5	1.34	17.7	1.06	14.0	1.10	14.6
Glu	11.43	0.86	7.5	0.84	7.3	0.54	4.8	0.53	4.6
Pro	8.08	-	-	-	-	-	-	-	-
Gly	3.08	0.23	7.3	0.20	6.6	0.16	5.3	0.16	5.1
Ala	2.44	0.46	18.8	0.37	15.1	0.29	11.8	0.24	10.0
½ Cys	-	-	-	-	-	-	-	-	-
Val	7.38	1.08	14.7	0.77	10.4	0.66	9.0	0.73	9.9

Met	2.11	1.65	78.0	1.42	67.5	0.92	43.7	0.95	45.1
Ile	6.01	0.65	10.8	0.54	9.1	0.39	6.5	0.39	6.5
Leu	12.31	5.59	45.4	6.00	48.7	4.35	35.3	4.22	34.3
Tyr	10.37	4.44	42.8	4.13	39.8	3.77	36.4	3.56	34.4
Phe	7.31	4.41	60.3	4.07	55.7	3.71	50.8	3.59	49.1
Try	2.50	1.75	70.0	1.73	69.1	1.83	73.3	1.41	56.6
TOTAL	100.66	30.24		27.49		23.17		21.02	

[a]Acid hydrolyzed native protein in 6N HCl for 22 h, values expressed as g/100 g digest.

[b]Values expressed as g/100 g digest.

[c]Values expressed as per cent of the theoretical total.

Table 7. Free amino acids in pepsin-pancreatin digest of β-lactoglobulin samples

Amino acid residue	Theoretical total[a]	Control		Heated		Heat + 1% glucose		Heat + 5% glucose	
		Digest[b]	(%)[c]	Digest	(%)[c]	Digest[b]	(%)[c]	Digest	(%)[c]
Lys	9.36	5.20	55.5	3.94	42.1	3.15	33.6	2.73	29.1
His	2.72	0.84	30.9	0.67	24.6	0.65	24.0	0.63	23.1
Arg	2.32	1.62	70.0	1.37	59.2	0.90	38.8	0.73	31.6
Asp	10.28	0.76	7.4	1.00	9.7	0.92	9.0	1.05	10.2
Thr-Ser	8.37	2.36	28.2	2.07	24.8	1.45	17.4	1.34	16.0
Glu	16.03	1.02	6.4	0.93	5.8	0.56	3.5	0.57	3.5
Pro	4.43	-	-	-	-	-	-	-	-
Gly	0.94	0.13	13.8	0.10	11.0	0.10	11.0	0.08	8.6
Ala	5.47	1.53	28.0	1.40	25.6	1.11	20.3	1.05	19.2
½ Cys	1.76	1.08	61.1	0.77	43.7	0.35	19.7	0.48	27.4
Val	5.81	2.50	43.0	2.19	37.7	1.77	30.5	1.88	32.3

Met	3.01	1.82	60.6	1.32	43.8	0.97	32.2	0.95	31.6
Ile	5.73	2.27	39.7	2.05	35.8	1.64	28.7	1.53	26.7
Leu	13.92	8.45	60.7	5.96	42.8	5.44	39.1	5.42	38.9
Tyr	3.88	2.14	55.2	1.85	47.7	1.56	40.3	1.49	38.4
Phe	3.82	1.64	42.9	1.35	35.3	1.03	26.9	1.04	27.1
Try	2.13	1.23	57.9	0.90	42.3	0.86	40.6	0.72	33.7
TOTAL	99.98	34.59		27.87		22.46		21.69	

[a]Acid hydrolyzed native protein in 6N HCl for 22 h, values expressed as g/100 g digest.

[b]Values expressed as g/100 g digest.

[c]Values expressed as per cent of the theoretical total.

Table 8. Decreases in enzymatically released free amino acids[a]

Amino acid residue	Whole casein			α_s-Casein			β-Lactoglobulin		
	Heat	Heat + 1% glucose	Heat + 5% glucose	Heat	Heat + 1% glucose	Heat + 5% glucose	Heat	Heat + 1% glucose	Heat + 5% glucose
	%								
Lys	7	12	48	19	38	61	24	39	48
His	b	22	45	b	7	23	20	23	25
Arg	22	46	37	9	45	55	15	44	55
Thr-Ser	-	-	-	-	-	-	-	-	-
Val	b	b	b	29	39	32	12	29	25
Met	5	7	24	14	44	42	27	47	48
Ile	b	b	b	17	40	40	10	28	33
Leu	b	b	b	b	22	25	29	36	36
Phe	b	b	b	8	16	19	18	36	36
Trp	b	28	19	b	b	19	27	30	42

[a]Values of the essential amino acids plus arginine are expressed as a percentage of the amount released in the untreated protein digest.

[b]Blanks indicate there was an increase in amount that was released.

Table 9. Comparison of the digest and residue fractions[a] in a pepsin-pancreatin digest of untreated milk proteins

Amino Acid	Whole egg		Whole casein		α_s-Casein		β-Lactoglobulin	
	Digest	Residue[b]	Digest	Residue[b]	Digest	Residue[b]	Digest	Residue[b]
Lys	2.68	4.42	3.36	4.41	4.03	0.40	5.20	4.16
His	0.78	1.97	0.77	2.51	0.86	1.76	0.84	1.88
Thr[c]		(4.64)		(3.80)		(2.28)		(4.57)
Val	1.77	5.01	0.62	6.19	1.08	6.30	2.50	3.31
Met + Cys	1.87	2.44	1.48	0.93	1.65	0.46	2.12	1.88
Ile	0.88	4.42	0.51	4.53	0.65	5.36	2.27	3.46
Leu	4.84	4.12	4.05	4.97	5.59	6.72	8.45	5.47
Phe + Tyr	5.84	3.56	4.70	4.30	6.91	6.90	3.78	3.68
Try	1.14	0.78	1.26	1.24	1.74	0.75	1.23	0.90
TOTALS	19.80	26.72	16.75	29.08	22.52	28.65	26.39	24.74

[a]Data expressed in mg/100 mg total amino acids recovered after acid hydrolysis.

[b]Determined by subtracting the digest values from those of the acid hydrolyzate.

[c]Threonine-serine peaks failed to resolve, values in parentheses represent total amounts present.

DISCUSSION

Static vs Dynamic System

A static digest is represented by a system where the substrate and enzymes are mixed and allowed to react to completion in a flask or beaker. Aliquots from the reaction mixture was extracted for analysis. The advantages are 1) ease and simplicity, 2) low cost and 3) not limited by equipment resource. The problems as outlined by Mauron (1955) are the possibilities of 1) end product inhibition and 2) the action of chymtrypsin as a transpeptidase.

A dynamic digest is represented by a system simulating the intestinal tract where the end products of enzymatic digestion are continually removed from the site of reaction. Mauron (1955) and Ford and Salter (1966) used different devices to achieve a dynamic digestion. The advantages appear to be that the system 1) more closely resembles the functioning of the digestive tract, it 2) avoids the disadvantages of a static digest, and 3) offers the possibility of having a nearly complete digestion. However, the disadvantages are 1) increased sophistication of instruments and manipulation, 2) higher cost, 3) a probable restriction on the number of samples

digested at one time, and 4) the current impossibility of removing only small peptides and/or free amino acids.

At the beginning of this study, a hollow fiber beaker "Osmolyzer" manufactured by Dow Chemicals was tested in a dynamic mode. Because the results of the study supported discontinuing its use, the findings have been relegated to incorporation into the Appendix.

Arai et al. (1975) claims to have induced a transpeptidase activity with chymotrypsin by using a 30% solution of oligopeptides. The driving force of the reaction is the extreme scarcity of free water. Diehl (1975) proposed that hydrophobic aggregation of the peptides represents a more realistic mechanism for the apparent increase in peptide weight. Therefore, at the 1% concentration of peptides in this study, there is no indication that chymotrypsin acts as a transpeptidase.

This study has shown that free amino acids do inhibit the proteolytic action of pancreatin on whole protein substrate. Because inhibition is competitive in nature, the adverse effects can be partially overcome by lengthening the time of reaction and/or by increasing the enzyme concentration. Since pancreatin loses two-thirds of its activity after 4 h at 37 C, the latter option was selected.

Akeson and Stahmann (1965) digested whole egg and casein with 15 mg of pepsin and 40 mg of pancreatin/g

protein. The amount of free amino acids recovered for whole egg and casein was 24 mg and 16.5 mg/100 mg digestion mixture, respectively. In his dynamic system, Mauron (1970) digested whole egg with 12.5 mg of pepsin and 25 mg pancreatin/g protein. The amount of free amino acids recovered was 22 mg/100 mg of digestion mixture. Under the static conditions of this study, whole egg and whole casein were digested by 83 mg of pepsin and two additions of 100 mg portions of pancreatin/g protein. Twenty-five mg and 22 mg of free amino acids were released/100 mg digestion mixture from the whole egg and whole casein digestion, respectively. Hence, the dynamic system used by Mauron (1970) fails to release any higher percentage of free amino acids than a static system. This study's high levels of enzyme compared to the amounts used by Akeson and Stahmann (1965) also failed to release significantly more amounts of free amino acids. However, the advantage to using the higher levels of enzyme is the subsequent breakdown of large peptides to smaller ones (see Table 2 and Figure 2). This further digestion aids greatly in simplifying the preparative steps necessary for analysis by an amino acid analyzer. Akeson and Stahmann's (1965) procedure required deproteinization with picric acid (which destroyed tryptophan), further separation of the large peptides by gel filtration, and then evaporation in order to obtain a proper concentration.

Although researchers (e.g. Sheffner et al., 1956; Akeson and Stahmann, 1964; and Mauron, 1970) seemed quite hesitant about using large dosages of enzyme, in vivo enzyme secretions, according to Nixon and Mawer (1970a), range from 66 to 280 mg/g ingested protein. The argument, Menden and Cremer (1966), favoring low enzyme additions in vitro was the speculation that large amounts of enzyme without a substrate would undergo autolysis, thus producing a digestion unrepresentative of the enzyme's behavior in the presence of a substrate. From the results of this study, increasing the enzyme concentrations does not initiate an increase in autolytic behavior. Rather, the increase in enzyme produces an increase in response (absorptivity or free amino acids) which is proportional to its concentration and the very slight proteolytic autolysis which occurs is uniform irrespective of the amount of enzyme.

Proteolytic Digestion of Milk Proteins

Strucutral Differences

Using the amount of free amino acid released as a gauge of digestibility, β-lactoglobulin, α_s-casein, and whole casein ranked from most to least. Probably, their structural difference attribute to the observed differences. Mellander (1955) recognized that an enzymatic digest of casein left a considerable residue of high molecular

weight peptides which he attributed to phosphorylated residues. McKenzie (1967) noted that α_s-casein's disordered configuration would allow for rapid hydrolysis. Tam and Whitaker (1972) found the initial rates of peptic hydrolysis on α_s-casein was twice that of whole casein. Fox and Guiney (1973) observed that α_s-casein was quite susceptible to proteolysis, but that its susceptibility decreased in heterogenous aggregated systems. Tanford et al. (1962) indicated β-lactoglobulin has a compact native structure but can refold following denaturation and produce a large number of α-helicies.

Effect of Treatments

Reduction in amounts of amino acids released in the heat treated proteins may result from 1) blocking, 2) destruction, and 3) tertiary rebonding leading to segments resistant to hydrolysis. Many investigators (e.g. Bender, 1972; Hurrell and Carpenter, 1974; and Groux, 1974) cite the amino acids with charged side groups as potential sources of interaction with each other or with compounds such as reducing sugars. Lien and Nawar (1974a, b) identified some thermal decomposition products of alanine, leucine, isoleucine, and valine.

The effects of the treatments on the release of amino acids in the casein digest agree with those of Erbersodobler (1969), Menden and Cremer (1966) and Rao and Rao (1972). The principal exception would be

increased amounts of isoleucine and leucine released compared to significant decreases observed by others. The difference may be due to differences in type of enzymes used.

It would be worthwhile noting from Figures 6, 7, and 8 that the more easily digested proteins of β-lactoglobulin and α_s-casein experience degradation at a more extreme degree than does whole casein. Because all of the free amino acids released in the β-lactoglobulin and α_s-casein digests decreased systematically with increases in added glucose, it can be deduced that structural changes are significant and produce a molecular configuration which is resistant to hydrolysis. Mills and Creamer (1974) observed that heating of β-lactoglobulin solutions caused marked irreversible changes to both the orientation of the proteins backbone as well as the amino acid side chains. Nakanishi and Wada (1974) identified some decomposition products of heated β-lactoglobulin. Evidence of increasing amounts of furfurals, Samuelsson and Nielson (1970), and protein bound pigments, Groux (1974), would support the concept of the formation of large peptide segments more resistant to further enzymatic hydrolysis. Reducing sugar can condense with the functional side groups of lysine and arginine preventing trypsin from hydrolyzing the adjacent bonds.

Pepsin Pancreatin Digest Index

Sheffner et al. (1956) originally formulated the mathematical concept for analyzing an in vitro digest while he termed the Pepsin Digest Ratio (PDR). This index compared favorably to the protein's biological value. Akeson and Stahmann (1964) used both pepsin and pancreatin in their digestion mixture and termed their index Pepsin Pancreatin Digest (PPD) which followed the basic formula of Sheffner et al. (1956). Mauron's (1970) only alteration to the PPD was the incorporation of a dialysis apparatus, hence the index became Pepsin Pancreatin Dialysis Digest (PPDD). Both the PPD and PPDD show excellent correlation to the biological value of proteins studied.

The formula for the basic index considers separately the free amino acids and the rest of the digest, i.e., the residue. Both are compared to a similar digest of whole egg. Sheffner et al. (1956) gives a full description of the procedure. The data required to calculate PPD indicies are given in Table 9. Values calculated for casein, α_s-casein, and β-lactoglobulin were 78, 75 and 95, respectively, where egg protein was assumed to be 96.5. Published biological values for casein range from 69 (Block and Mitchell, 1946), 73 (Mitchell and Block, 1946), to 78 (Rippon, 1959).

The PPD indicies for the treated protein digests were not calculated due to Mauron's (1970) observation

that such a determination does not reveal a close approximation to animal assays. One explanation suggests that acid used to hydrolyze the digest in order to obtain the composition of the residue fraction releases amino acids that are not released in vivo. Simply using the free amino acids present in the digestion mixture to determine an index does not appear to yield a representative index. After incubating intestinal contents of a milk meal, Nixon and Mawer (1970b) suggested that the rate of liberation of glycine, proline, and the dicarboxylic acids was so slow that it was necessary to postulate absorption as peptides or hydrolysis at the mucosal surface.

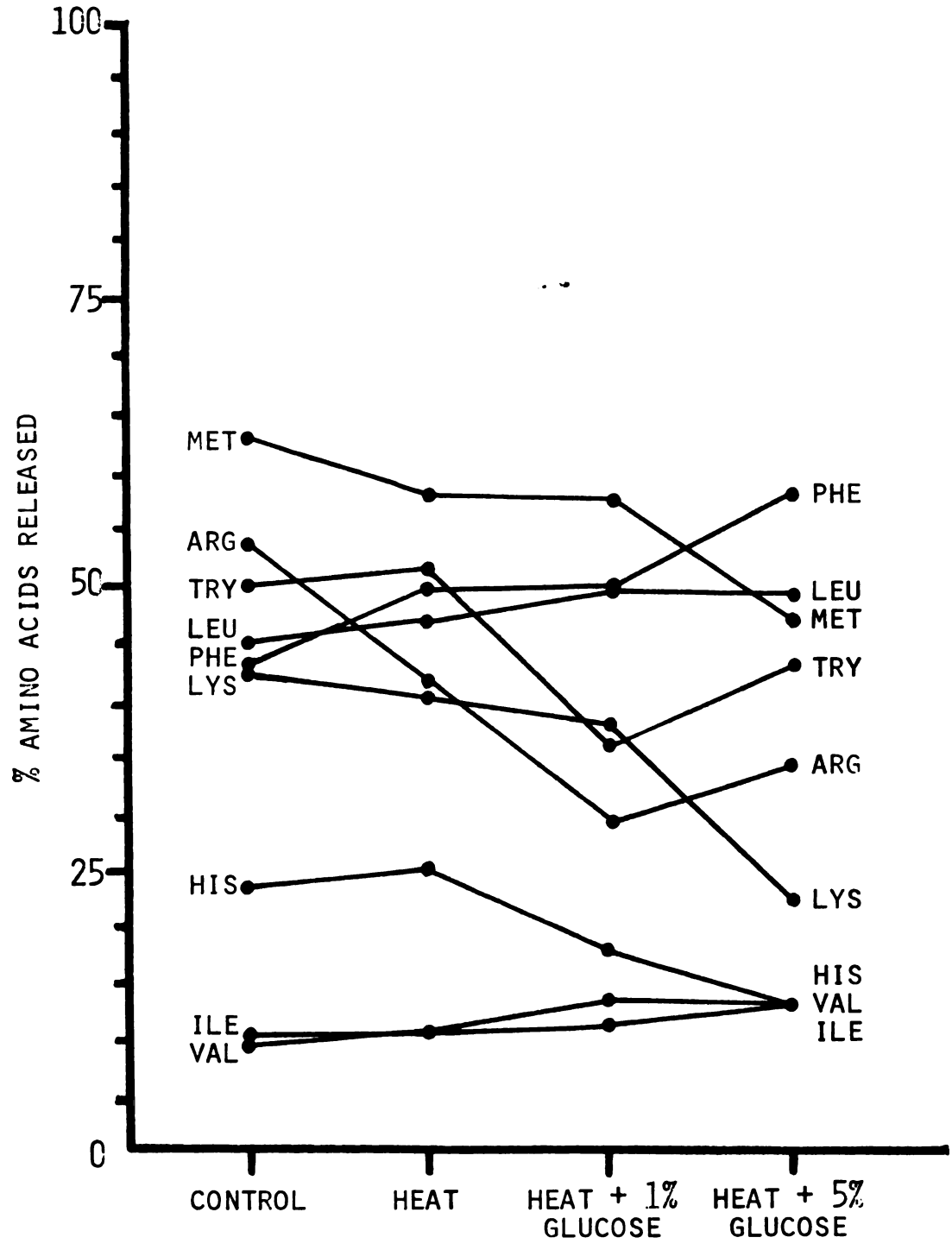

Figure 6. Effects of various treatments of whole casein on the enzymatic release of amino acids

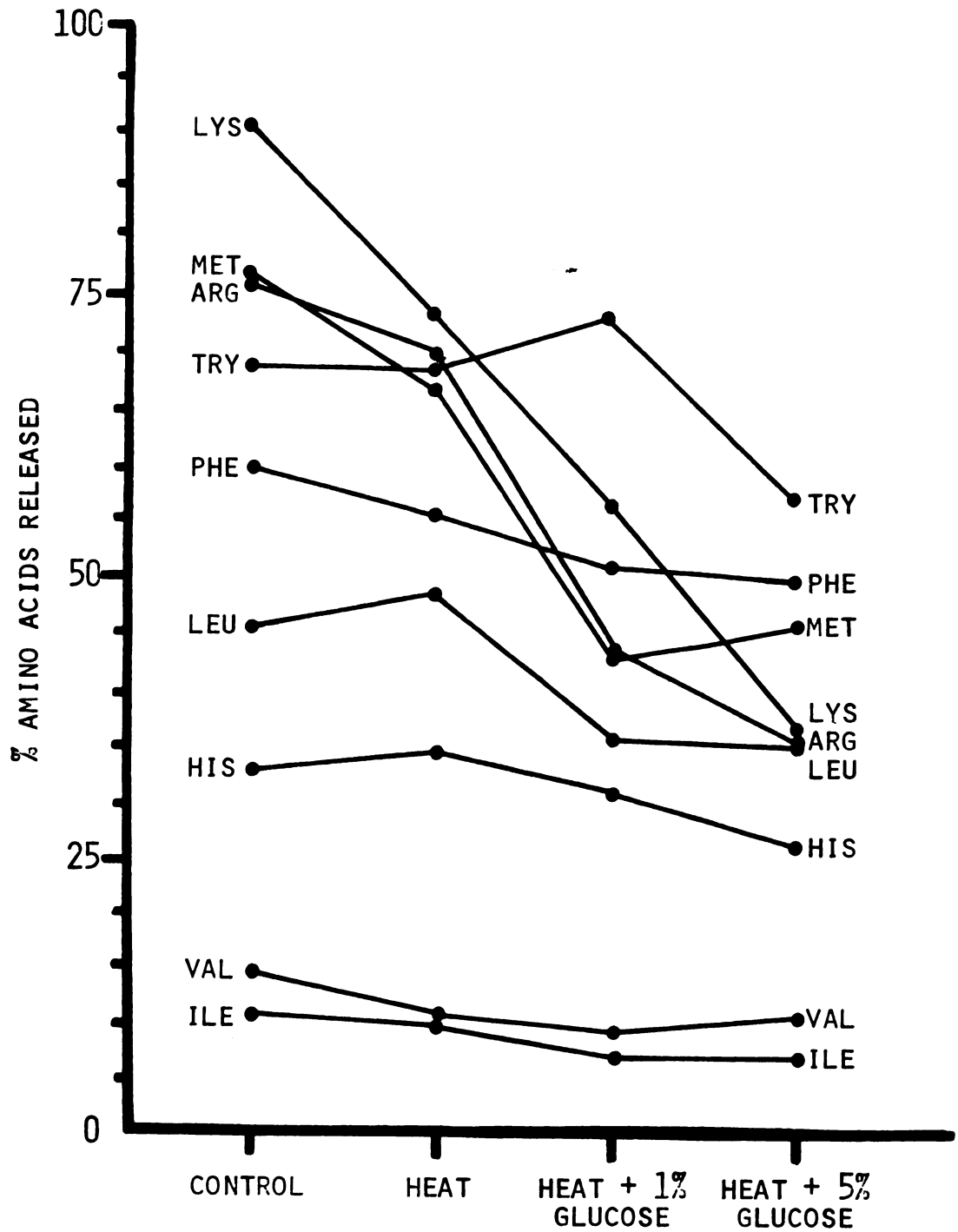

Figure 7. Effects of various treatments of α_s-casein on the enzymatic release of amino acids

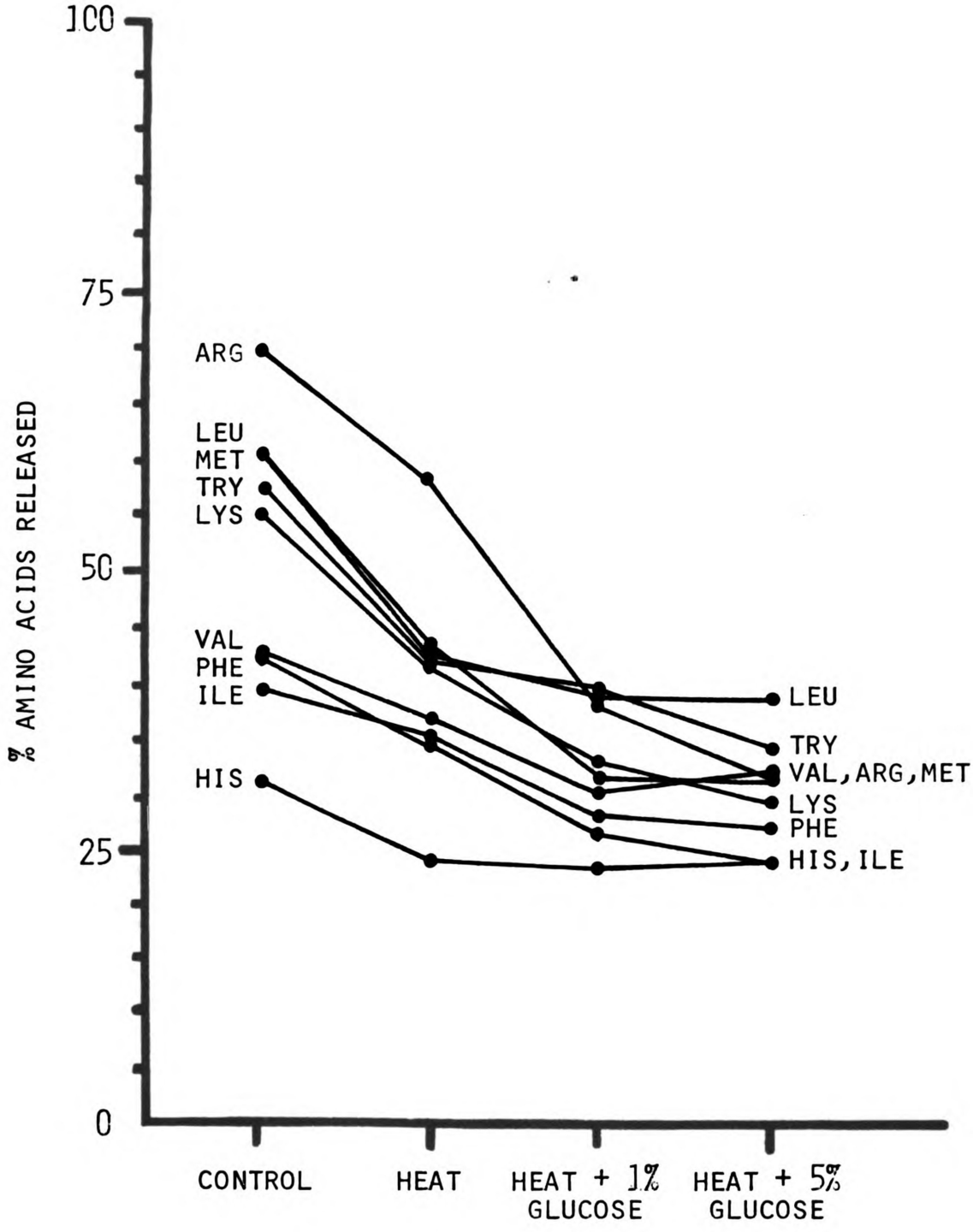

Figure 8. Effects of various treatments of β-lactoglobulin on the enzymatic release of amino acids

CONCLUSION

Differences can be noted between the three selected milk proteins and the effects of treatments on those proteins by using this particular method of *in vitro* enzymatic digestion. The authenticity of the data obtained from such a digest must be verified by animal studies. Once accomplished, this sensitive technique could offer invaluable application in the area of the development of protein foods. Investigators interested in 1) the effects of various processes on the protein's nutritive value, 2) the quality of new sources of protein for human food, and 3) the optimization of the contribution of several protein foods to the total diet would find the *in vitro* enzymatic digest an economical, rapid method for monitoring the protein's nutritive value.

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APPENDIX

Reagents for ninhydrin test

1. Citrate buffer contains 4.3 g citric acid and 8.7 g Na-citrate·$2H_2O$ in 250 ml, adjust to pH 5.
2. Add 400 mg $SnCl_2 \cdot 2H_2O$ in 250 ml citrate buffer (0.2 M) pH 5.
3. Add the above solution to 250 ml methyl Cellosolve containing 10 g dissolved ninhydrin.

Amino Acids Used for Pancreatin Inhibitor Study

Amino acid	mg/ml
glycine	5.28
L-arginine	5.27
DL-serine	5.22
L-alanine	6.26
L-histidine	5.68
L-lysine	5.40
DL-valine	4.61
DL-phenylalanine	4.84
DL-methionine	5.49
TOTAL	48.04

Dynamic Digest System

A "Beaker Osmolyzer" with cellulose acetate, hollow fibers, produced by Dow Chemicals, with a molecular weight cutoff of 200 was tested for use in an in vitro enzymatic digestion. The potential advantage of this apparatus was its capacity to remove free amino acids continuously from the site of digestion.

A concentration solution of $CaCl_2$ was passed through the tubing to remove the water and free amino acids from the beaker by reverse osmosis. To conduct further analysis of gross amino acids by the ninhydrin test, it was necessary to remove the Ca (II) from the diffusate. This was best accomplished by precipitating the Ca (II) as $Ca(OH)_2$ with NaOH at pH 12-14. The supernatant was readjusted to pH 5 by the addition of formic acid and pH 5 phosphate buffer. Table A lists the other reagents tested:

Table A. Applicability of various reagents for the removal of Ca (II)

Reagent	Observations
NH_4-oxylate	Good precipitory agent
HNa_2PO_4	Poor precipitory agent
$NaBO_3$	Poor precipitory agent
Na-oxylate	Highly insoluble
Na_2SO_4	Highly insoluble

The quickest method to detect the presence of individual amino acids proved to be by dansylation (Hartley, 1970). It was not essential to remove the Ca (II) in order to visualize the spots. The one requirement was to adjust the reaction mixture to pH 10-11. The NaOH treatment, previously described, caused an excessive DNS-OH reaction. Table B lists other procedures employed to yield clear, dansylated amino acid spots on polyamide sheets:

Table B. Effectiveness of various chemicals for the qualitation of amino acids in a Ca (II) solution by dansylation

Reagent	Observations
Triethylamine	Produced too large DNS-OH spot
$NaHCO_3$	Precipitated out with Ca (II)
NH_4-oxylate	Produced too large DNS-NH_2 spot
Acetone + $NaHCO_3$	Produced a drop in pH
Pryidine	Raised pH to only 8.4
Acetone + triethylamine at pH 10	Produced the best plate, reaction conditions were 2 h at 40 C

The amino acids lysine, glycine, histidine, phenylalanine, alanine, valine, and tyrosine were observed to pass through the tubing. Not all 18 amino acids were tested, e.g. the carboxylic acid group. The major drawback to the hollow fiber device was the poor rate of transfer of the amino acids and preferential transfer

rates. With a mixture of the water soluble amino acids in the beaker, two concentrations of $CaCl_2$ were passed through the tubing. The percentage of total amino acids and water recovered was determined by the ninhydrin test after 50 min. Using a 7% $CaCl_2$ solution, 2.4% amino acids in 7% of the water was recovered. With a 15% $CaCl_2$ solution, 17.5% amino acids in 25% of the water was recovered. Qualitiative determinations by dansylation showed the recovery was proportional to the amino acids remaining. The poor rate of recovery of the amino acids merited its discontinuation as a viable mode for a dynamic digestion. Chloride ions passed into the beaker from the tubing network, which, potentially, could inhibit the activity of trypsin, a component of pancreatin.

Enzymatic Digestion

of the Dried, Whole Egg Solids Sample

Amino acid residue	Literature[a]	Theoretical[b]	Digest[c]	%[d]
Lys	6.6	7.1	2.7	37.8
His	2.3	2.8	0.8	28.6
Arg	6.4	6.6	3.1	47.0
Asp	9.7	9.8	0.2	2.0
Thr	4.8	4.6	1.1	9.1
Ser	7.1	7.4		
Glu	12.6	12.6	0.5	3.9
Pro	3.7	4.7	-	-

Gly	3.1	2.8	-	-
Ala	5.5	5.0	0.6	12.0
½ Cys	2.3	1.9	0.3	15.8
Val	6.1	6.8	1.8	26.5
Met	3.3	2.4	1.6	66.6
Ile	5.0	5.3	0.9	17.0
Leu	8.2	9.0	4.8	53.4
Tyr	4.0	4.1	2.7	65.8
Phe	4.8	5.3	3.1	58.5
Try	1.9	1.9	1.1	58.0
TOTAL	97.4	100.0	25.2	

[a]Values stated as g/16 g N after acid hydrolysis, Mauron (1970).

[b]Values used in this study, stated as g/100 g total amino acids recovered by acid hydrolysis.

[c]Digested with pepsin-(2X)pancreatin used in this study, values expressed as g/100 g digest.

[d]Values expressed as percent of the theoretical total.