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J. Robert Brunner
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COMPARISON OF IN VITRO ENZYMATIC DIGESTION AND RAT BIOASSAY (PER) PROCEDURES FOR ASSESSMENT OF THE NUTRITIONAL QUALITY OF THERMALLY AND CHEMICALLY MODIFIED CASEIN

By

Helen C. McCune

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ABSTRACT

COMPARISON OF IN VITRO ENZYMATIC DIGESTION AND RAT BIOASSAY (PER) PROCEDURES FOR ASSESSMENT OF THE NUTRITIONAL QUALITY OF THERMALLY AND CHEMICALLY MODIFIED CASEIN

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The purpose of this study was to compare protein quality results as measured by a rat bioassay (PER) with those obtained using an in vitro enzymatic digestion procedure. Vitamin free casein was treated by four different thermal and chemical procedures in order to provide experimental samples varying in protein quality. Using these test proteins, three different parameters of an in vitro enzymatic (pepsin-pancreatin) digestion were investigated. These included: the initial rate of digestion, the relative proportions of free amino acids and peptides in the products of digestion, and analyses of the amino acids released.

The most sensitive indicator of nutritional quality of the proteins was the profile of amino acids released during digestion. Various indices based on the essential amino acids released were calculated and compared to the PER results. The Enzyme Index correlated better than either the Pepsin Pancreatin Digest Index or the Enzyme Score with the PER results.

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INTRODUCTION

New and proposed government regulations concerning the nutritional value of protein foods has renewed interest in the investigation of new methods to determine protein quality. Currently, the method used to determine the protein quality for nutritional labeling information is the Protein Efficiency Ratio (PER). This method is time consuming and expensive. Moreover, there are numerous theoretical limitations associated with the PER method.

Because of the recent use of proteins from unconventional sources as food ingredients, new processing techniques have been developed. Thus, there is a need for a rapid method which correlates well with the official method, PER, to monitor any loss in protein quality during processing.

In response to the above needs, various chemical and enzymatic methods have been developed to provide rapid, less expensive procedures for protein quality determination. These methods include: Chemical Score, Pepsin Digest Residue Index, and Pepsin Pancreatin Digest Index. However, more work is needed before these procedures can be used as a replacement of protein quality evaluation.

The object of this research was to compare a rat bioassay with in vitro enzymatic methods. Various aspects of the enzymatic digestion were investigated to determine if they reflected the nutritional value of the protein. These included: the initial rate and extent of digestion and the examination of the amino acids released by enzymatic digestion.

LITERATURE REVIEW

Protein Quality Determination

Since the advent of nutritional labeling and other government regulations concerning protein foods, the quantitative study of protein quality has received added impetus. The Protein Efficiency Ratio (PER) is the legal analytical basis for protein nutritional labeling (Code of Federal Regulations, 1977). Nesheim (1977) has reviewed the use of PER in nutritional labeling and many proposed FDA regulations. The current regulation states that the USA Recommended Daily Allowance (RDA) of a protein is set at the level of 45 g if the PER of the protein is equal to or greater than the PER of casein; and 65 g if the PER of the protein is less than that of casein (Code of Federal Regulations, 1977). Furthermore, a protein with a PER which is less than 20% of the PER for casein must be labeled "not a significant source of protein." Other proposed regulations concerning fortification of foods, formulated meal replacements, fortified ready-to-eat or hot cereals, plant protein products, and the addition of amino acids to foods all are based on PER determination. The PER method is time consuming and

has many disadvantages both in terms of cost and methodology. Bodwell (1977) pointed out that proteins with a PER of less than 2.4 and greater than 0.5 will be treated as if they were nutritionally equivalent in these regulations; obviously they are not. Therefore, the food industry and concerned scientists are endeavoring to develop faster and more reliable methods of assessing protein quality.

Bioassays

The evaluation of protein quality by animal bioassay has been the subject of several reviews (Hegsted, 1977; Hegsted, 1974; Hackler, 1977). In an ideal bioassay, the amino acid content of the protein, the bioavailability of each amino acid, and the amino acid requirement of the test animal should be known qualities. At the same time, the relationship between an animal's response to a protein and a human's response should be recognized. These facts, however, have not been entirely elucidated. Moreover, there are a myriad of other factors which have been known to influence bioassays, including: age, sex, and weight of the animal, protein quality and quantity, food intake, other dietary components such as carbohydrate, minerals, vitamins, fat, and water, husbandry, and environmental conditions.

Mitchell (1924) utilized the principle of nitrogen balance to develop a method suitable for determining

protein quality. His index, Biological Value (BV), is defined as a ratio of retained nitrogen to absorbed nitrogen of a test animal. This method is technically cumbersome because of the difficulty in collecting feces and urine, and in accurately estimating the endogenous urinary and fecal nitrogen (Williams et al., 1974). Another drawback of this method is that there is no allowance for digestibility. The development of the Net Protein Utilization (NPU) (Bender and Miller, 1953) and Net Protein Ratio (NPR) (Bender and Doell, 1957) techniques eliminated some of the difficulties associated with the BV method. Both NPU, which is defined as carcass nitrogen of a test group minus the carcass nitrogen of a protein-free group divided by the nitrogen consumed, and NPR, represented by the weight gain of a test group plus the weight loss of a protein-free group divided by nitrogen consumed, include protein digestibility as part of the assessment. Although NPU and NPR are technically less rigorous than BV, the carcass nitrogen determination can present problems. In the calculation of NPR, the assumption that the body composition is constant may not be valid when widely different test and protein deficient diets are fed to the animals. The major drawback with the above methods is the dependence of the results on the level and quality of dietary protein. For example, the BV of meat, oats, corn, and

potatoes is lower for a diet containing 10% protein than it is for a diet containing only 5% protein. This difference in apparent BV is smaller with the poorer quality proteins such as potatoes and oats (McLaughlin, 1972).

Hegsted and co-worker (1965, 1969) developed an assay relating nitrogen balance or weight gain to nitrogen intake. In this procedure a protein is fed at different levels; the slope of the growth response curve is an indication of protein quality. However, the slope may not always be a valid index of protein quality. For example, if lysine is the limiting amino acid, a lower than expected slope is obtained; and if the limiting amino acid is threonine, a higher than expected slope is noted. In addition, it is sometimes difficult to select the linear portion of the response curve.

The PER method (Osborn and Mendel, 1919) which is defined as the ratio of weight gain over protein consumed by a weanling rat for a 28-day test period, is probably the simplest bioassay. There are, however, some fundamental problems associated with PER as an index of protein quality. No allowance is made for amino acid maintenance requirements of the test animal. It is also difficult to measure complimentary effects of two or more proteins, when using PER as an indication of protein quality. In addition to BV, NPU, and NPR, McLaughlin (1972) found that PER, too, varied with the amount of

protein in the diet. For example, the highest PER for egg protein is obtained when the protein is fed at the 8% level. In contrast, for wheat protein the highest PER is observed when protein is fed at a 15% level.

Since PER is the basis of nutritional labeling, various researchers have studied factors which might affect the results (Morrison and Campbell, 1960; Hurt et al., 1975; Hegarity, 1975; Steinke, 1977). The results of these studies indicated that: (1) younger rats (21-23 day old versus 29 day old) yield a higher PER; (2) different rat strains vary in their PER response, but standardization of test results with respect to the apparent PER of casein eliminates the problem; and (3) female rats grow slower than males thus producing different PER values. Studying the effects of the nonprotein dietary components, Hurt et al. (1975) determined that the mineral content and dietary fat composition (saturated versus unsaturated) did not affect the PER. However, the level of dietary fat and fiber significantly influenced the results. Fiber and fat were found to affect the PER of casein to the same extent as the test protein. Therefore, if both the casein and test diets are balanced with respect to these ingredients, the results will not be biased. Steinke (1977) discussed the effect of different carbohydrates on PER. He found that rats prefer a sweeter tasting diet. Consumption of

a sweet diet will exceed that of an unsweetened diet of similar composition. Hopkins and Steinke (1976) found that the hydration of a protein prior to mixing improved the PER. Interestingly, the hydration of soy isolate raised the PER to a greater extent than the hydration of casein.

More recently bioassays have been developed to eliminate some of the problems associated with PER. McLaughlin and Keith (1975) developed a modification of the PER method which incorporated the maintenance requirement into the calculation. In this method, Nitrogen Utilization (NU) is defined as weight gain plus 0.1 times the initial weight plus final weight divided by the protein consumption. NU is often expressed as Relative Nitrogen Utilization (RNU) when it is compared to a standard which is generally lactalbumin.

Another new method which includes the maintenance requirement in the value for protein quality is the Weight Gain Coefficient (Canolty and Koong, 1977). The slope of the line which correlates weight gain, expressed as kg of body weight per metabolic body size, and feed consumption is defined as the Weight Gain Coefficient. More data on these methods are indicated before they could replace PER as the standard method. These assays are not rapid; requiring 3-4 weeks.

Chevez and Pellet (1976) compared several bioassays for twelve different food mixtures common to Latin America. These included Relative Protein Value (RPV) (slope assay calculated without zero protein data), Relative Nutritive Value (RNV) (slope assay calculated with zero protein data), PER, and NPR. Regardless of the assay employed, all the dietary proteins were similarly ranked (see Table 1). In another comparison, McLaughlin and Keith (1975) determined the PER, RNU, slope assay, and NPR for twelve different proteins of varying protein quality. They found similar values for protein quality by each method, except that the PER values tended to underestimate the quality of the lower quality proteins.

Chemical Methods

Other methods used to evaluate the nutritional quality of proteins have been based on the chemical analysis of the amino acid content of the protein. Mitchell (1946) based his method on the fact that all amino acids required for the synthesis of proteins must be present at the site of synthesis; and that protein synthesis would be limited by the essential amino acid present in the least supply. His assessment of protein quality, Chemical Score, is represented by the smallest ratio of an essential amino acid of a test protein to the same amino acid in whole egg protein. Egg protein

Table 1. Comparison of bioassay techniques to determine protein quality (from Chavez and Pellet, 1976).

Food	First Set of Samples				
	RNV	RPN	PER	NPR	Relative NPR
Lactalbumin	1.00	1.00	4.00	4.60	1.00
Rice, Beans	0.85	0.95	3.00	3.70	0.81
Cassava, Beans, Plantain, & Cheese	0.85	0.99	2.80	3.70	0.81
Corn, Beans	0.70	0.74	2.10	3.10	0.68
Bread, Cheese, & Beans	0.70	0.77	2.00	3.10	0.66
Spaghetti, Cheese, & Tomato Sauce	0.64	0.68	1.80	2.90	0.62
Arepa & Margarine	0.42	0.28	0.50	1.90	0.42
Food	Second Set of Samples				
	RNV	RPN	PER	NPR	Relative NPR
Lactalbumin	1.00	1.00	4.50	4.80	1.00
Arepa, Meat	1.04	1.14	4.50	4.80	1.00
Arepa, Sardines	0.87	0.94	3.90	4.20	0.86
Arepa, Cheese	0.86	0.92	3.80	4.10	0.83
Rice, Plantain, & Sardines	0.82	0.75	3.60	3.80	0.79
Rice, Beans, & Plantain, Meat	0.76	0.80	3.30	3.60	0.75
Arepa, Beans	0.69	0.76	2.90	3.30	0.67

has been found to have excessive amounts of certain amino acids relative to the requirements of growing rats. In particular, tryptophan, and to some extent, the sulfur amino acids are in excess; however, lysine is present at about the requirement level. Therefore, if the limiting amino acid is lysine, the Chemical Score and PER will be proportional (McLaughlin, 1972).

Because of the excess of essential amino acids in the egg pattern, the FAO/WHO (1973) proposed a provisional amino acid pattern based on the amino acid requirements of pre-school infants. The index of protein quality using this pattern of amino acids is referred to as the Amino Acid Score. Kaba and Pellet (1975) compared various amino acid scoring patterns (whole egg protein, human milk protein, whole rat carcass, FAO, 1957, and FAO/WHO, 1973). The FAO/WHO (1973) pattern was superior to the other patterns in predicting the limiting amino acids for test diets in which the first limiting amino acids were either lysine or methionine. In addition, the correlation between amino acid score, using the 1973 pattern, and NPU with rats was higher than when other patterns were used.

Oser (1951) devised a criterion for protein quality, Essential Amino Acid Index, based on the comparison of the essential amino acids of a test protein to the essential amino acids of whole egg protein. In

contrast to the Chemical Score, which is merely the expression of the smallest ratio, the Essential Amino Acid Index is the geometric mean of all the ratios of the essential amino acids of the test protein to that amino acid of the reference protein. This index is generally higher than the Chemical Score.

The major problem with chemical evaluations of protein quality is the assumption that all the amino acids are biologically available; an assumption which is not always valid. On the other hand, the advantages of these indices are: (1) the small sample size required, (2) the short time of analysis compared to bioassays, and (3) the information provided concerning the identity of the limiting amino acid.

In Vitro Enzymatic Methods

Development of enzymatic digestion methods to assess protein quality began when Melnick et al. (1946) reported that different proteins, or proteins which had been processed differently, were hydrolyzed by pancreatin at different rates. Evans and Butt (1949) found that by autoclaving soya, the release of some of the amino acids by enzymatic digestion was retarded.

Mauron et al. (1955) examined the availabilities of lysine, methionine, and tryptophan in different milk products by analyzing the amino acids liberated by in

vitro enzymatic digestion. The milk products were digested with pepsin for 14 h followed by pancreatin for an additional 24 h. The digestion was run in a dialysis apparatus in which the end products were constantly being removed in order to avoid any possible end product inhibition.

As an improvement over the evaluation methods which only measured digestion rate or amino availability, Sheffner et al. (1956) employed an index based on an in vitro digestion with pepsin to measure protein quality. They used sufficient pepsin to obtain approximately 30% hydrolysis of the test protein. This index was based on the assumption that the initial products of digestion represented an indication of the nutritional value of the protein. After pepsin digestion, the samples were deproteinated and the released essential amino acids were determined by microbiological methods. A Pepsin Digest Residue Index (PDRI) was then calculated by comparing the released essential amino acids and those remaining undigested with the pattern of essential amino acids found when whole egg protein was digested in a similar manner. The calculation also involved correction for the difference in degree of proteolysis between the test and reference protein. For the proteins tested, the BV using rats and PDRI/digestibility were highly correlated.

The microbiological determination of the amino acids for the PDRI was slow and laborious. In 1964, Akeson and Stahmann used an amino acid analyzer to determine the essential amino acids released during digestion with pepsin and pancreatin. They used the same calculations as the PDRI to obtain a Pepsin Pancreatin Digest Index (PPDI). The results of the two indices were similar. The suggested reason for the similarity in the results of the two indices was that during the microbiological determination of amino acids some peptides were utilized by the microbes; thus, increasing the apparent amount of free amino acids. In the PPDI, the use of the pancreatin released an equivalent amount of free amino acids as the microbial utilization (Mauron, 1970).

Ford and Salter (1966), like Mauron, were concerned about end product inhibition. Therefore, they digested samples on a column of Sephadex G-10 to provide for chromatographic removal of small digestion products (less than 700 daltons). After digestion and deproteination, the samples were fractionated on a Sephadex G-25 column. Amino acid analysis was done of the fraction with a molecular weight of less than 250. Comparison of the amino acid results of the static digests of Akeson and Stahmann (1964) and the dynamic digestion methods of Ford and Salter (1966) and Mauron (1970) revealed

no apparent differences. These data indicated that end product inhibition was inconsequential to these assays (Stahmann and Woldegiorgis, 1975).

PPDI, PDRI, and NPU were used to monitor the effect of heat processing casein (Stahmann and Woldegiorgis, 1975). It was observed that all three methods predicted similar values for protein quality. An Enzyme Score, which is calculated like the Chemical Score, comparing the essential amino acids released from the test protein and those released from egg protein, was also determined on the heat processed casein. The Chemical Score and Enzyme Score compared well with the PPDI. However, the Chemical Score tended to underestimate the quality of the most severely heated protein.

The major problem with the use of in vitro digestion methods as an evaluation of protein quality concerns the incomplete digestion of the test protein. Therefore, a reference protein is always assayed in conjunction with the test protein. The advantages of using the in vitro enzymatic digestion methods for deriving protein quality indices include: (1) lower cost and shorter analysis time, (2) less variation in in vitro results than found with rat bioassays, (3) the generation of information on amino acid availability and relative nutritional adequacy (Stahmann and Woldegiorgis, 1975).

More recently, Satterlee et al. (1977) employed a simplified in vitro digestion to develop a value for predicting PER. These authors have used a recording pH meter to determine the extent of digestion by trypsin, chymotrypsin, and petidase. The pH after 10 min was found to correlate with apparent digestibility determined in vivo. A combination of the digestibility and the essential amino acids content of the protein, expressed as a percentage of the FAO/WHO-1973 provisional pattern, was used to derive an apparent PER. The actual rat PER and the apparent PER were compared for 45 different food samples. The average difference between the estimates was 0.12 PER units.

Protein Damage

The effect of processing on protein quality has been reviewed by Bender (1972). He classified protein damage into four categories: (1) amino acid destruction by oxidation, (2) loss of palatability, (3) modification of some linkages, or (4) formation of enzyme resistant linkages. The extent of damage is usually dependent on food composition and processing conditions.

Pure Protein

In the preparation of protein isolates and the texturization of vegetable protein, the temperature of processing often exceeds 100 C. Bjarnason and Carpenter

(1970) studied the mechanism of damage of pure proteins. After heating bovine plasma albumin 27 h at 115 C, the cysteine and lysine contents were reduced. At 145 C for 27 h, all the amino acids except glutamic acid and those with paraffin side chains were reduced. These authors suggested that the loss of lysine was due to the reaction of its ε-amino group with the amide group of asparagine and glutamine. In addition, the ε-amino group of lysine appeared to react with the decomposition products of cysteine.

A number of researchers have investigated the nutritional significance of the lysine-containing peptides formed during heat treatment in the absence of carbohydrate or fat (Ford and Shorrock, 1971; Warbel and Carpenter, 1972; Hurrell and Carpenter, 1976). Their results indicated that the formation of the lysine-containing peptides do not totally explain the low biological value of the heated protein. Ford (1973) suggested that the formation of lysine-glutamine bonds may hinder the access of peptidases to adjacent bonds in the protein.

Severe heat treatment of casein (1-8 h at 120-130- C) induced a decrease in aspartic acid, threonine, serine, cysteine, histidine, and lysine. In contrast, glutamic acid and alanine were found to increase after the heat treatment (Osner and Johnson, 1974). The BV and NPU of casein, which had been heated under the same

conditions as above, decreased significantly (Osner and Johnson, 1975). The microbiological availability of valine, methionine, isoleucine, leucine, histidine, arginine, and tryptophan decreased after the heat treatment. In addition, the in vitro pepsin digestibility of the heated casein was found to decrease after severe heat processing (Osner and Johnson, 1968).

Protein and Carbohydrate

The Maillard reaction, or nonenzymatic browning, has been studied extensively (Bender, 1972; Janicek, 1973; Feeney et al., 1975; Adrian, 1974). In food products, generally the amino groups from proteins and aldehyde groups from either reducing sugars or carbonyl compounds formed during lipid oxidation are the reactive species. The series of reactions results in brown pigments and a destruction of certain amino acids (see Figure 1). The loss of amino acids occurs long before melandoidin pigments are formed. The first reaction product, an aldosamine, is not considered to be nutritionally available. Although it is hydrolyzed with concentrated acid, the aldosamine is not hydrolyzed during digestion (Feeney et al., 1975). For example, in over-heated milk, unavailable lysine was found to be in either the aldosamine, Schiff base, or deoxy-ketosyl form. After acid hydrolysis, lysine in the aldosamine or Schiff base was regenerated, but only 49.5% of the lysine in the deoxy-ketosyl

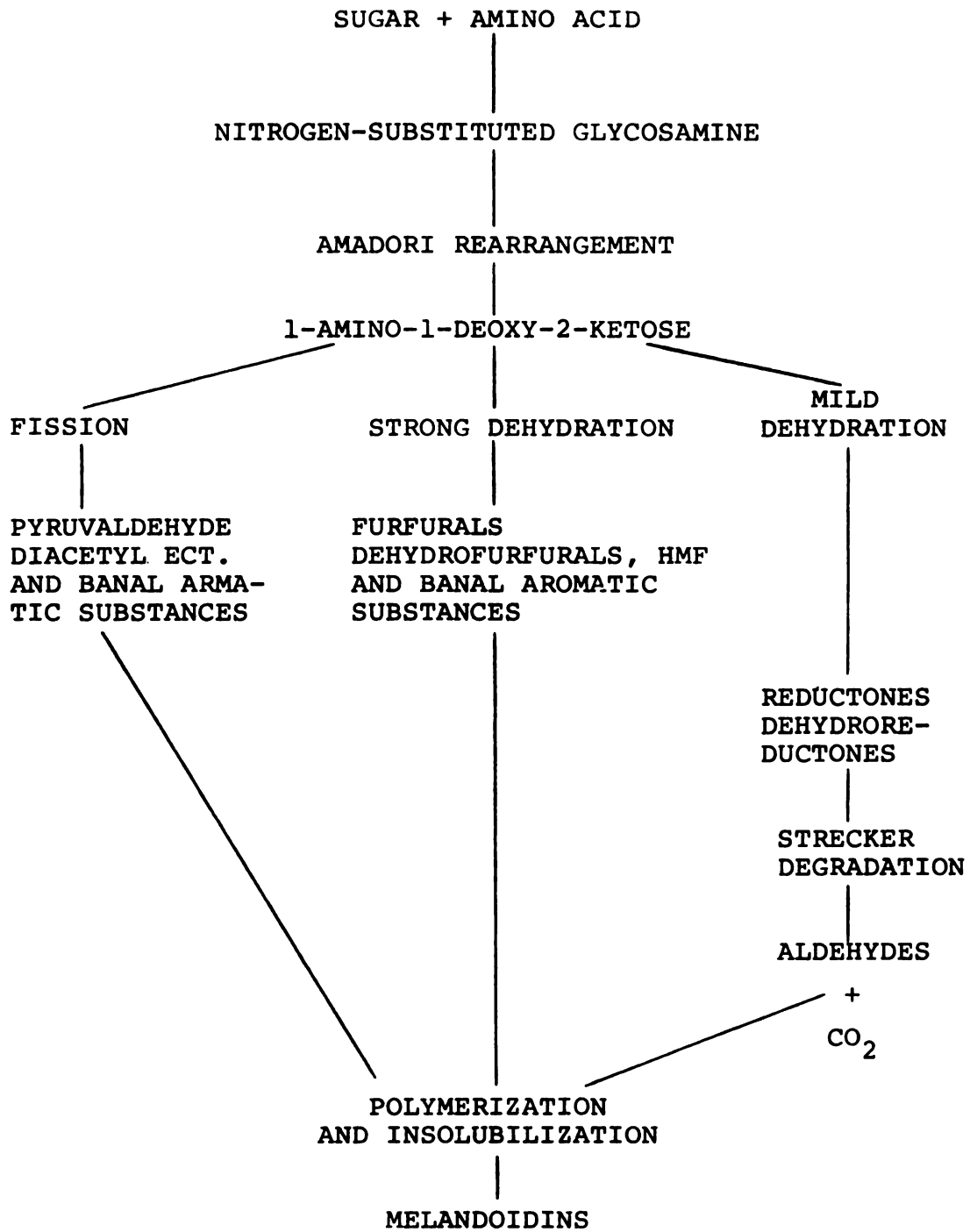

Figure 1. Principle stages of the Maillard Reaction (from Adrian, 1974)

form was recovered (Finot, 1973). Tanaka et al. (1975) examined the metabolism of fructose-L-tryptophan, formed after Amadori rearrangement, using rat feeding studies. While a portion of the fructose-L-tryptophan could be absorbed intestinally, most of the compound was excreted in the urine without being metabolized. The nutritional effects of the Maillard reaction were well illustrated by the results of Baldwin et al. (1951) who observed that the PER of a casein-glucose model system, which had undergone some browning during autoclaving for only 3 min, decreased by 25%.

The Maillard reaction can occur during storage, and certain conditions increase the rate of the reaction. Browning increases with temperature, is favored by alkaline conditions, and is strongly dependent on water activity (Bender, 1972). The type of sugar influences the rate of the reaction; pentoses being more reactive than aldoses (Tu and Eskin, 1973).

The amino acids most affected by the Maillard reaction are generally the N-terminal chain amino acids, followed by the basic amino acids, the sulfur amino acids, and sometimes tryptophan (Feeney et al., 1975). The reaction between the ε-amino group of lysine and reducing sugars has been extensively examined by Carpenter (1973). In addition to the amino acids mentioned above, histidine, and threonine are subject to heat

degradation in the presence of carbohydrate (Bender, 1972; Baldwin et al., 1951; Ford and Salter, 1966).

Protein and Lipids

The presence of oxidizing lipid can also cause damage to proteins. The destruction of sulfhydryl groups in either amino acids or SH enzymes occurred more rapidly in the presence of oxidized than unoxidized linoleic acid (Lewis and Wills, 1962). Methionine, histidine, cysteine, and lysine were found to be particularly sensitive to free radicals produced by peroxidizing lipids (Roubal and Tappel, 1966). Andrews et al. (1965) investigated the reaction between insulin and oxidizing methyl linoleate. They found that the intermediates of fatty acid oxidation react with the ε-amino groups of lysine and the N-terminal amino acids, phenylalanine and glycine.

Porkony (1973, 1975) examined the browning of proteins which were exposed to oxidizing lipids. Tannenbaum et al. (1969) found that the methionine content of casein, which had been mixed with methyl linoleate and incubated for 67 days, decreased as the browning increased.

Malonaldehyde (MA), a dialdehyde intermediate of fatty acid oxidation, appeared to react with amino acids and form fluorescing compounds (Karel, 1973). When reacting the protein myosin, MA was found to selectively attack histidine, arginine, tyrosine, and

methionine (Buttkus, 1967). Crawford et al. (1967) studied the mechanism and kinetics of the reaction between bovine serum albumin and MA. The N-terminal amino acids and the amino group of lysine were the reactive sites on the protein. When ribonuclease was incubated with oxidizing lipid, MA was implicated in the inactivation of the enzyme (Chio and Tappel, 1969).

The nutritional consequences of the protein lipid interaction have been studied. For example, the PER for herring meal was observed to decrease from 2.94 to 2.53 after lipid oxidation had occurred (Carpenter et al., 1963). Other researchers have studied the effects of oxidized ethyl linoleate on casein nutritional quality (Yangita et al., 1973). The in vitro digestibility of casein decreased after the reaction with oxidized ethyl linoleate. The available lysine of casein decreased after incubation with an oxidized fatty acid. Lysine and methionine were destroyed to the greatest extent (Horigome et al., 1974; Horigome and Muira, 1974). When egg albumin and oxidized ethyl linoleate were combined, BV and true digestibility decreased. Histidine, lysine, arginine, and methionine were reduced to the greatest extent after exposure of the egg albumin to oxidized ethyl linoleate (Yanagita and Sugano, 1974).

EXPERIMENTAL

Chemicals and Materials

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

Other chemicals from various commercial sources were employed and will be mentioned in the description of the method where used.

Preparation of Protein Model Systems

Vitamin-free casein (INC Pharmaceuticals) was used in all the following model systems:

1. Casein-Glucose Model System

A 25% (w/v) glucose solution was prepared using deionized water. When the glucose was completely dissolved, sufficient casein was added to produce a slurry which was approximately 50% total solids, containing equal parts of glucose and protein. The mixture was mixed vigorously in a Warring Blender and freeze dried.

2. Casein-Safflower Oil Model System

A 20% (w/v) safflower oil (PVO International Inc.) mixture was prepared with deionized water and homogenized at 2000 psi with a Chase-Logeman Laboratory Homogenizer. Employing a Warring Blender, sufficient casein was blended into the safflower-oil-water mixture to yield a 40% total solids slurry, containing equal quantities of safflower oil and protein. In order to insure rapid oxidation of the oil, 0.2 ppm of copper in the form of cupric sulfate was added. The mixture was blended vigorously and freeze dried.

A copper-free casein-safflower oil model system was prepared as above.

Model System Treatments

1. Casein-Safflower Oil Model System (minus copper)

Immediately after freeze drying, the casein-safflower oil mixture was extracted twice with hexane (1 part to 5 parts hexane), and one time with diethyl ether (1 part to 5 parts diethyl ether). After extracting the oil from the system, the residual casein remained under the hood for approximately 12 h to exhaust residual ether and placed in vacuum oven (30 mm Hg pressure) for 5 h at ambient temperature to insure that all traces of solvent were removed.

2. Casein-Safflower Oil Model System (with copper)

After freeze drying, the casein-safflower oil combination was incubated 10 days at 55 C to promote oxidation of the oil. Following the incubation period, the oil was extracted as described above.

3. Casein

Casein was spread approximately 1-2 in. thick in Pyrex pans which were covered with aluminum foil. The casein was then autoclaved for 5 min at 121 C (15 psi).

4. Casein-Glucose Model System

The casein-glucose mixture, after freeze drying, was autoclaved in the same manner described for casein.

Enzymatic Digestion

Both treated and untreated proteins were partially dissolved in deionized water by slowly adjusting the pH to 1.8 with 1 N HCl. Sodium azide was added at the 0.02% level to prevent microbial growth. The volume was adjusted with deionized water to obtain a 1% protein solution. Thirty ml of the 1% protein solutions were added to 125 ml flasks along with 15 mg pepsin. After the pepsin was dissolved, the flasks were stoppered and placed in a 37 C incubator for 3 h.

Peptic digestion was terminated by the addition of 1N NaOH until pH 8.3 was reached. Deionized water was added to make the total addition of liquid equal to 5 ml. Then 15 mg of pancreatin was added to each flask.

The contents of the flasks were mixed and reincubated at 37 C. After 3 h of pancreatin digestion, a second 15 mg portion of pancreatin was added and the digestion continued for an additional 21 h.

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

Chemical Methods

Preparation of Digestion Samples for Analyses

The digestion of the samples was stopped by adding sufficient trichloroacetic acid (TCA) to obtain a final concentration of 15%. After sitting overnight at 4 C, the samples were centrifuged in a clinical centrifuge at 1000x g for 5 min to remove the precipitated material. The clear supernatant was analyzed for nitrogen, alpha amino nitrogen, and free amino acids.

Nitrogen-Micro Kjeldhal

Approximately 15 mg of dried protein were digested in duplicate with 4 ml of digestion mixture over a gas flame for 1 h. The digestion mixture contained 5.0 g $CuSO_4$:$8H_2O$ and 5.0 g SeO_2 in 500 ml of concentrated H_2SO_4. After cooling the flasks, 1 ml of 30% H_2O_2 was added, and digestion was continued for an additional hour. Each digestion flask was then cooled, and the sides of the flask were rinsed with 10 ml of deionized water. The digestion mixture was neutralized

with 25 ml of a 40% NaOH solution. The released ammonia was steam-distilled into 15 ml of a 15% boric acid solution containing 5 drops of bromocresol green, methyl red indicator, containing 400 mg bromocresol green and 40 mg methyl red in 100 ml 95% ethanol. The distillation was continued until the volume of the boric acid receiver reached 60 ml. The ammonium borate complex was titrated with 0.02 N HCl which had been standardized with a standardized solution of NaOH. The recovery of tryptophan N served as a control. Nitrogen was calculated as follows:

$$\%N = \frac{(\text{ml HCl} - \text{ml blank})\ (\text{Normality of HCl}) \times 100}{\text{mg of sample}}$$

Alpha Amino Nitrogen-
Ninhydrin Test

The method of Clark (1964) for the determination of alpha amino nitrogen was followed. Aliquots (0.5 ml) of deproteinated diluted samples were pipetted into test tubes. The dilution factors ranged from 10, 25, to 100 for the samples from the enzyme blank, peptic digest, and second pancreatin digest, respectively. A reagent blank was run with 0.5 ml of deionized water. Then 1.5 ml of ninhydrin solution (see Appendix for preparation) was added to each tube. The contents of the tubes were mixed well, and placed in a boiling water bath for 20 min. After heating, the tubes were cooled and 8 ml of a 50% (w/v) aqueous n-propanol solution was

added to each tube. The contents were mixed vigorously. After 10 min, the absorbance was read at 570 nm against the reagent blank on a Beckman D K 2 A Double Beam Spectrophotometer. A standard curve was prepared using glycine.

Amino Acids--Acid Hydrolysates

Amino acid analyses were performed on HCl hydrolysates of protein using a Beckman Amino Acid Analyzer, Model 120 C, according to the procedures of Moore et al. (1958). Samples consisting of approximately 4 mg of protein were weighed into 10 ml ampoules. Five ml of 6 N HCl were added to the ampoules. The contents were frozen in a dry-ice-ethanol bath. The ampoules were evacuated with a high vacuum pump. As the contents slowly melted, the gases were removed. The contents were then refrozen and the ampoules were sealed using an air-propane flame. The sealed ampoules were placed in an oil bath in a forced draft, recirculating oven regulated at 110 ± 2 C for 24 or 48 h.

After hydrolysis, the ampoules were opened, and 1 ml of norleucine solution (2.5 μmoles/ml) was added as an internal standard. The hydrolysate was then quantitatively transferred from the ampoule to a 25 ml pear-shaped flask. The hydrolysate was evaporated to dryness on a rotary evaporator. The dried sample was washed with a small amount of deionized water and again taken

to dryness. In all, three washings were performed to remove residual HCl. The washed and dried hydrolysate was dissolved in 0.067 M citrate HCl buffer (pH 2.2), and diluted to a volume of 5 ml. The solution was then filtered with a 0.22 μm Millipore Filter, and 0.2 ml aliquots were used for analysis. The chromatograms were quantitated by peak integration using a Spectra Physics Autolab System AA. Standard amino acid mixtures were analyzed using the same ninhydrin solution within a four-day period.

Methionine and Cystine Analysis

Since methionine and cystine undergo a variable amount of oxidation during acid hydrolysis, they must be analyzed separately. The methods of Schram et al. (1964) and Lewis (1966) were used. These methods involve performic acid oxidation of methionine and cystine to methionine sulfone and cysteic acid, respectively. Approximately 10-20 mg of sample, representing 5-8 mg of protein, was weighed into a 25 ml pear-shaped flask. The protein was oxidized for 24 h with 10 ml of performic acid at 4 C. After oxidation, 1 ml of norleucine (2.5 μmoles/ml) was added. The performic acid was removed on a rotary evaporator. The dried sample was quantitatively transferred to a 10 ml ampoule with 5 ml of 6 N redistilled HCl. Hydrolysis and amino acid analyses were performed as previously discussed.

Free Amino Acids--Enzymatic Hydrolysates

The 27 h peptic-pancreatin digestions were deproteinated with 15% TCA. After centrifugation to remove the precipitated protein, the supernatant was filtered with 0.8 μm Millipore Filter. Then, 2 ml of the filtrate plus 1 ml of norleucine solution (2.5 μmoles/ml) were diluted to 5 ml with 0.67 M citrate HCl buffer (pH 2.2). Because the enzyme blank contained much less nitrogen, a 12 ml sample plus 1 ml of the norleucine solution was evaporated to dryness on a rotary evaporator. The dried sample was then diluted to 5 ml with the same citrate buffer. The diluted sample was filtered with a 0.22 μm Millipore Filter. Amino acid analyses were performed as previously described. Since acid hydrolysis was not employed, the sulfur amino acids were not destroyed and could be quantitated directly.

Tryptophan

The method of Spies (1967) was followed. This method involved a 24 h pronase (Sigma Chemical Co.) digestion, followed by the production of a chromaphore resulting from the action of free tryptophan with p-dimethylaminobenaldehyde (Matheson, Coleman, Bell Co.). Absorbance of the sample was determined against a reagent blank at 590 nm on a Beckman D K 2 A Double Beam Spectrophotometer. A pronase blank was also run so that

samples could be corrected for the tryptophan content of the pronase. A standard curve was prepared with tryptophan which had been dried over P_2O_5.

In the case of the free amino acids, the same procedure was followed except that the pronase digestion step was eliminated.

Thiobarbituric Acid Test (TBA)

The extent of lipid oxidation in the casein-safflower oil model system with copper was monitored with a modified TBA test procedure of King (1962). A 1.00% protein solution was prepared with deionized water. The mixture was blended in a Warring Blender on high speed for 3 min. Aliquots (15 ml) were placed in small flasks. Two ml of aldehyde-free redistilled ethanol and 2 ml of 50% TCA were added, followed by vigorous shaking. The samples were held for 30 min and filtered through Whatman no. 1 and no. 42 filter paper, consecutively. Four ml of each filtrate was pipetted into a screw-cap test tube. One ml of TBA reagent was added to each, followed by mixing and incubation in a 60 C water bath for 1 h. After cooling, the absorbance of the sample was read versus distilled water at 532 nm on a Beckman D K 2 A Double Beam Spectrophotometer. See Appendix for description of reagent preparation.

Peroxide Value

The peroxide value was determined according to the AOAC (1975) method. To approximately 5 g of oil sample, 30 ml of an acetic-acid-chloroform (3:2) mixture was added. Then, 0.5 ml of a saturated KI solution was added, followed by 30 ml of deionized water. The solution was titrated with a standardized 0.1 N $Na_2S_2O_3$ solution. The peroxide value, expressed as meq. peroxide/kg sample was calculated as follows:

$$\frac{(\text{ml of } Na_2S_2O_3)\ (\text{N of } Na_2S_2O_3) \times 1000}{\text{g sample}}$$

Moisture

Approximately 1 g samples were dried in a vacuum oven (30 mm Hg pressure) at 90-95 C for 20 h. The water loss was determined gravimetrically.

Bioassay--Protein Efficiency Ratio (PER)

The PER method of AOAC (1975) was followed.

Preparation of Diets

Five diets were prepared using either the untreated or treated casein samples. The composition of the diets is shown in Table 2. Each diet was thoroughly mixed in a Hobart Blender.

Table 2. Composition of diets used in PER test.

Ingredient	Diet[1]				
	1	2	3	4	5
Treated casein[2]					
Untrt	11.03				
Exunox		11.23			
Exox			12.90		
Auto				10.60	
Auto Glu					19.42
Cottonseed oil	8.00	8.00	8.00	8.00	8.00
Salt mix[3]	5.00	5.00	5.00	5.00	5.00
Vitamin mix[4]	1.00	1.00	1.00	1.00	1.00
Cellulose (Alphacell)[5]	1.00	1.00	1.00	1.00	1.00
Water	4.22	3.82	4.10	4.49	3.96
Corn starch	69.75	69.95	68.00	69.91	61.62

[1]Expressed in percent. Each diet contains 10% protein and 5% water. See Appendix for N and moisture contents of casein samples.

[2]Vitamin free casein from ICN Pharmaceuticals. Untrt, Untreated casein; Exunox, casein after extraction of unoxidized safflower oil; Exox, casein after extraction of oxidized safflower oil; Auto, casein autoclaved 5 min 121 C; Auto Glu, casein and glucose autoclaved 5 min 121 C.

[3]USP XVII, ICN Pharmaceuticals.

[4]Vitamin Diet Fortification Mixture, ICN Pharmaceuticals.

[5]ICN Pharmaceuticals.

Bioassay Method

After a 4-day acclimatization period on a standard casein diet, male, weanling, Spraque-Dawley rats were randomly assigned to 5 groups of 10 rats each. Rats were housed individually in metal cages with raised wire mesh floors. Water and diets were fed ad libum. Food intake and body weight were recorded at 3- and 4-day intervals, respectively, for the 28-day test period. PER (weight gain/protein intake) was calculated from the weight gain and protein consumption of each rat.

Statistical Analysis

The Analysis of Variance with a completely randomized block design and the Tukey procedure were used to analyze the data (Neter and Wasserman, 1974).

pH Stat Evaluation--Initial Rate of Digestion

Initial rates of pancreatin and trypsin digestions were determined on either intact proteins or pepsin pretreated proteins using a Sargent pH Stat.

Preparation of Protein Samples

A 1% protein solution, using either the treated or untreated casein samples, was prepared with 0.005 M TRIS, 0.04M NaCl buffer (pH8.3). For the pepsin pretreated proteins, a 1% protein solution was adjusted to pH 1.8. Pepsin was then added in the same enzyme to

substrate ratio (1:60) as that employed in the in vitro enzymatic digestion described above. After 3 h of digestion at 37 C, the pH was raised to 8.3 with 1 N NaOH, and the sample was ready for initial rate determinations.

Preparation of Enzyme Solutions

The pancreatin solution was prepared with 0.01 M HCl, 0.01 M $CaCl_2$ buffer (pH 3.8) to a concentration of 7 mg/ml. This solution was kept in an ice bath until ready for use. Sufficient pancreatin solution for 1 h of experimentation was adjusted to pH8.3 with concentrated NaOH and placed in an ice bath until needed. The trypsin solution (5.6 mg/ml) was prepared with the same buffer that was employed for the pancreatin solution. Since a much smaller amount of trypsin was used in the assay, no pH adjustment was required prior to use.

Initial Rate Determinations

Seven ml of protein solution, either intact or pepsin pretreated samples, were added to the reaction vessel. When the temperature reached 37 C and a stable base line was achieved, the enzyme was injected into the vessel. One ml of the pancreatin solution was used for the determination of the pancreatin initial rate. For the tryptic digestion, the amount of trypsin solution used was experimentally determined so that the initial rate of the tryptic digestion of the untreated casein

equalled that rate obtained when pancreatin was used. This amount was 0.75 µl of trypsin solution. A magnetic stirring bar maintained continuous mixing while the titrant, 0.1 M TRIS, was added. The initial rates, expressed as ml of base delivered/min, were determined by calculating the slope of the tangent to the reaction curve at zero time.

The data were analyzed using Analysis of Variance with a completely randomized block design and the Tukey procedure (Neter and Wasserman, 1974).

Gel Filtration Chromatography

A Sephadex G-25 column (2.6 cm x 36 cm) was prepared according to Pharmacia Fine Chemical (1974). The column was equilibrated with 0.1 M ammonium acetate buffer (pH 7.0) with 0.1% sodium azide. A void volume of 75 ml was determined using Blue Dextran.

RESULTS

Lipid Oxidation of the Casein-Safflower Oil Model System

When the casein-safflower oil model system with copper was incubated for 10 days at 55 C, the oil became quite oxidized as indicated by increased peroxide and TBA values (Table 3). The peroxide value rose by a factor of 100. The TBA values, which measures the fatty acid oxidation intermediate, malonaldehyde (MA), doubled. After the oxidized oil had been extracted, the TBA value of the extracted casein was still slightly higher than that of either the casein-safflower oil model system before incubation or the untreated casein. This indicates that possibly some MA was still present after solvent extraction.

Color Changes During Treatments

During the oxidation of the oil, the model system turned a light amber color which remained following solvent extraction. The autoclaved casein-glucose mixture turned a light tan color. However, autoclaving casein alone caused no color change.

Table 3. Peroxide values of safflower oil and TBA values of casein-safflower oil model system.

Sample	Peroxide Value (meq. peroxide/ kg sample)	TBA Value (Absorbance 532 nm)
Safflower oil before incubation	1.36	--
Safflower oil extracted from casein-safflower oil model system after incubation 10 days 55 C	159	--
Casein-safflower oil model system before incubation	--	0.055
Casein-safflower oil model system after incubation 10 days 55 C	--	0.133
Casein after extraction of oxidized oil	--	0.064
Untreated casein	--	0.044

Protein Efficiency Ratio Determination (PER)

The growth curves of the 5 groups of rats fed diets containing either untreated or the various treated caseins are presented in Figure 2. Those fed diets containing casein exposed to the oxidizing oil (Exox) grew at the slowest rate. Rats fed either an untreated casein (Untrt) diet, or the diet containing casein which had the unoxidized oil removed (Exunox) grew at approximately the same rate. When the diets with the autoclaved casein (Auto or Auto Glu) were fed, rats grew at a rate slightly less than that obtained when rats were fed the Untrt casein diet.

The PER results are presented in Table 4. Although the Exox casein exhibited the only PER which was significantly lower than the PER of the Untrt casein, the PERs for the auto-samples were lower than the Untrt, suggesting that heat treatment had a slight effect.

Amino Acid Content of Treated Proteins (Acid Hydrolysates)

Table 5 represents the total amino acid content of the 5 casein samples determined after acid hydrolysis for 24 or 48 h. These data represent the higher of the two values (24 or 48 h hydrolysis) for each amino acid. The various treatments had only a slight effect on the amino acid content. Because the precision of the amino

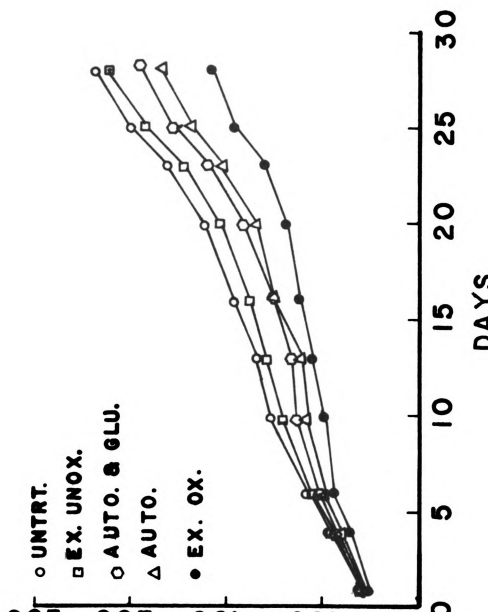

Figure 2. Average weight gain of rats fed diets containing either treated or untreated casein.

Table 4. Average weight gain, food consumption, protein intake, and protein efficiency ratio for rats fed either treated or untreated caseins.[1]

Casein Treatment[2]	Average Weight Gain	Food Consumed	Protein Intake	PER[3]
Untrt	135 ± 6	416 ± 10	42.4 ± 1.0	3.07 ± .08[ac]
Exunox	129 ± 5	400 ± 12	40.8 ± 1.2	3.14 ± .04[a]
Exox	77 ± 7	323 ± 13	32.9 ± 1.4	2.30 ± .12[b]
Auto	105 ± 8	360 ± 16	36.8 ± 1.6	2.83 ± .13[ac]
Auto Glu	115 ± 7	397 ± 15	40.8 ± 1.5	2.78 ± .07[c]

[1]Mean ± SEM.

[2]Untrt, No treatment; Exunox, Casein after extraction of unoxidized safflower oil; Exox, Casein after extraction of oxidized safflower oil; Auto, Casein autoclaved 5 min 121 C; Auto Glu, Casein and glucose autoclaved 5 min 121 C.

[3]Means not showing common superscript are significantly different ($P < 0.05$).

Table 5. Amino acid composition of treated and untreated caseins (expressed as g residue/16 g N).

Amino Acid	FAO[1]	Untrt[2]	Exunox[2]	Exox[2]	Auto[2]	Auto Glu[2]
Lys	5.5	6.61	6.85	7.04	7.07	6.18
Thr	4.0	3.85	3.78	3.72	3.89	3.80
1/2 Cys	3.5	0.35	0.39	0.36	0.39	0.40
Met		2.40	2.43	2.20	2.10	2.11
Val	5.0	6.99	6.46	7.21	7.48	6.98
Ile	4.0	5.05	4.87	5.08	4.87	4.93
Leu	7.0	8.67	8.20	9.58	8.39	8.66
Tyr	6.0	5.55	5.20	5.19	5.26	5.23
Phe		4.73	4.66	4.78	4.75	4.95
Trp	1.0	1.62	1.77	1.41	1.59	1.91
His		2.42	2.41	2.51	2.55	2.68
Arg		3.23	3.22	3.28	3.24	3.39
Asp		6.43	6.36	6.32	7.11	6.37
Ser		4.93	4.97	4.86	4.91	4.93
Glu		20.94	20.31	20.43	20.83	20.89
Pro		10.23	12.31	10.16	9.56	10.36
Gly		1.48	1.51	1.45	1.46	1.56
Ala		2.56	2.55	2.55	2.53	2.58
Ammonia		1.96	1.74	1.87	2.01	2.09

[1]FAO/WHO provisional pattern (1973).

[2]Untrt, No treatment; Exunox, Casein after extraction of unoxidized safflower oil; Exox, Casein after extraction of oxidized safflower oil; Auto, Casein autoclaved 5 min 121 C; Auto Glu, Casein and glucose autoclaved 5 min 121 C.

acid analyzer is ± 3%, only values which differ from the Untrt casein by greater than 3% are considered to be due to processing effects. In the Auto Glu sample, the lysine and sulfur amino acids decreased by 6% and 9%, respectively. Tryptophan and sulfur amino acid contents of the Exox casein were reduced by 6% and 12%, respectively. Sulfur amino acids were also decreased in the Auto casein; however, the aspartic acid and valine contents were slightly higher than those of the Untrt control. In the Exunox sample, valine and leucine were reduced by 7% and 6%, respectively.

The FAO/WHO (1973) provisional amino acid pattern used for scoring food patterns also appears in Table 5. By comparing this pattern with the amino acid contents of the 5 casein samples, the limiting essential amino acids can be estimated. In all 5 proteins, the sulfur amino acids and threonine were the first and second limiting amino acids, respectively. The Protein Score, which is the smallest ratio of an essential amino acid of the test protein to that amino acid in the provisional pattern, was calculated using the sulfur amino acids. The Protein Scores were Untrt, 79; Exunox, 81; Exox, 73; Auto, 71; Auto Glu, 72.

Another index of protein quality, the Essential Amino Acid Index, was calculated using the essential amino acid data of the 5 samples. The Essential Amino

Acid Index was calculated by computing the geometric mean of all the ratios of the essential amino acids of the test protein to those of the provisional pattern for each sample. The calculated indices were 97 for the Untrt and Exunox casein, and 95 for the Exox, Auto, and Auto Glu caseins.

In Vitro Enzymatic Digestion

Rate of Digestion

The rates of the in vitro enzymatic digestion for the 5 protein samples were obtained by measuring the alpha amino nitrogen of the TCA-soluble fractions at various times during the 27 h digestion (Figure 3). The amount of alpha amino nitrogen representing a 100% hydrolysis was estimated by determining the difference in alpha amino nitrogen in TCA supernatants obtained from acid hydrolyzed and unhydrolyzed casein. By comparing the alpha amino nitrogen content of the TCA supernatant from the digestion to the value representing 100% hydrolysis, the extent of hydrolysis was estimated.

The Untrt casein was digested at a higher rate than the other samples. The Auto sample was digested at a rate approximately equal to that of the Untrt, except that the final hydrolysis was slightly lower. Although the Auto Glu casein was hydrolyzed at a slow rate initially, by the end of the digestion, the extent of hydrolysis was nearly equal to that of the Auto sample.

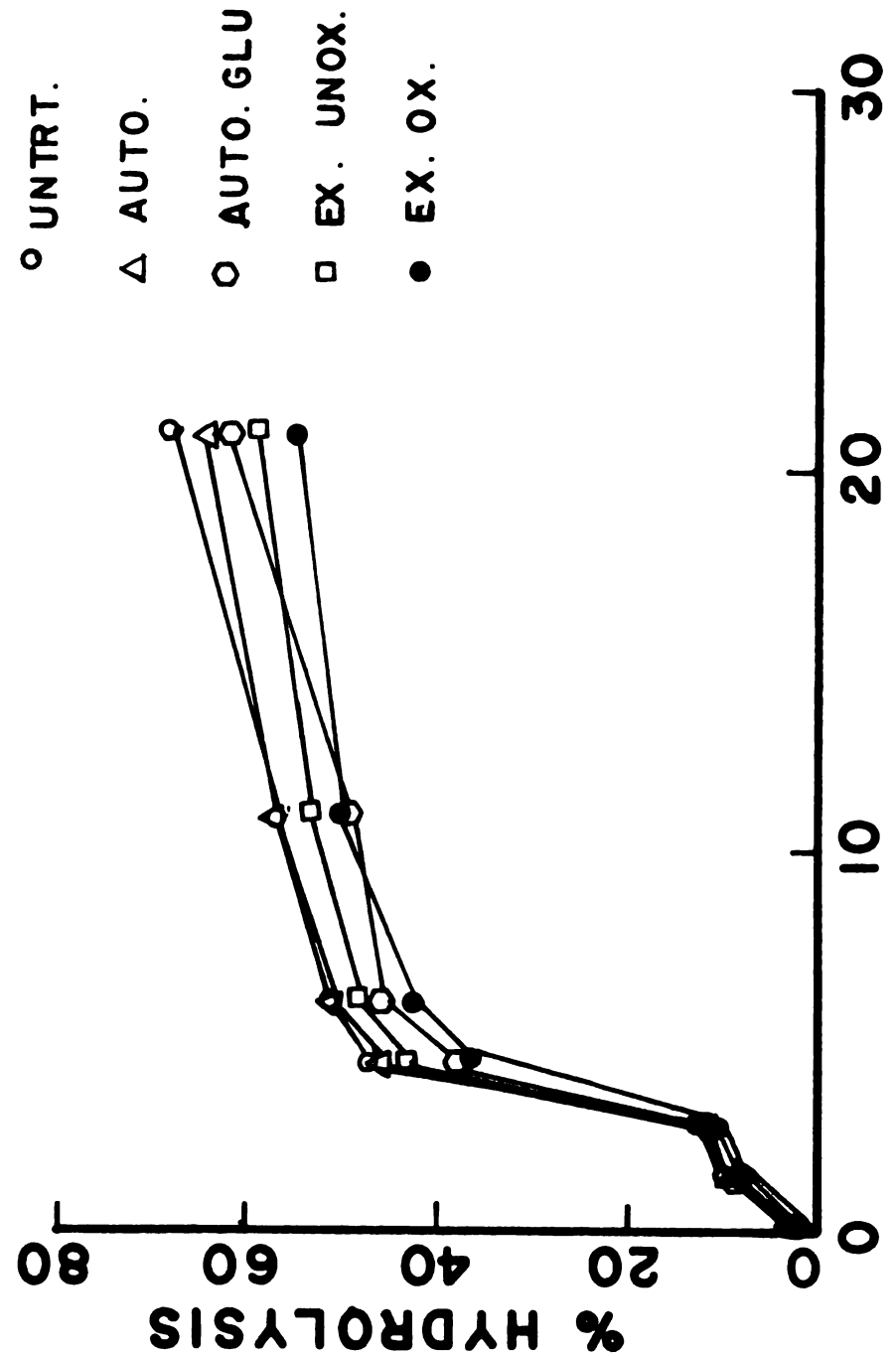

Figure 3. Rates of digestion of treated and untreated casein by pepsin-pancreatin-pancreatin.

The Exox casein was hydrolyzed at the slowest rate throughout the period of digestion. The rate of digestion of the Exunox casein was slightly greater than that of the Exox casein.

Gel Filtration of Digestion Products

The TCA soluble fractions from the 27 h digests were chromatographed on a Sephadex G-25 column (exclusion limit = 5,000 daltons) to determine their molecular size distribution. Gel filtration chromatograms are displayed in Figure 4. The slightly different elution volumes observed are due to small variations in flow rates. The first peak resolved on the chromatograms represents the void volume. Tryptophan, tyrosine, and TCA standards were chromatographed to determine their elution volumes. The elution volumes of the tryptophan and tyrosine standards correspond to peaks 6 and 7, respectively, on the chromatograms. Peak 4 on the chromatograms corresponds to the elution volume for the TCA standard. Based on these observations, no significant differences among the samples in the proportions of free amino acids and peptides could be detected.

Free Amino Acid Profile

The amino acids liberated by the in vitro pepsin-pancreatin sequential proteolysis of the treated and

Figure 4. Chromatograms from a Sephadex G-25 column of TCA-material from in vitro enzymatic digestion of treated and untreated caseins. a, untreated casein; b, casein after extraction of unoxidized safflower oil; c, casein after extraction of oxidized safflower oil; d, casein autoclaved 5 min 121 C; e, casein and glucose autoclaved 5 min 121 C.
1 Trichloroacetic acid Standard
2 Tyrosine Standard
3 Tryptophan Standard

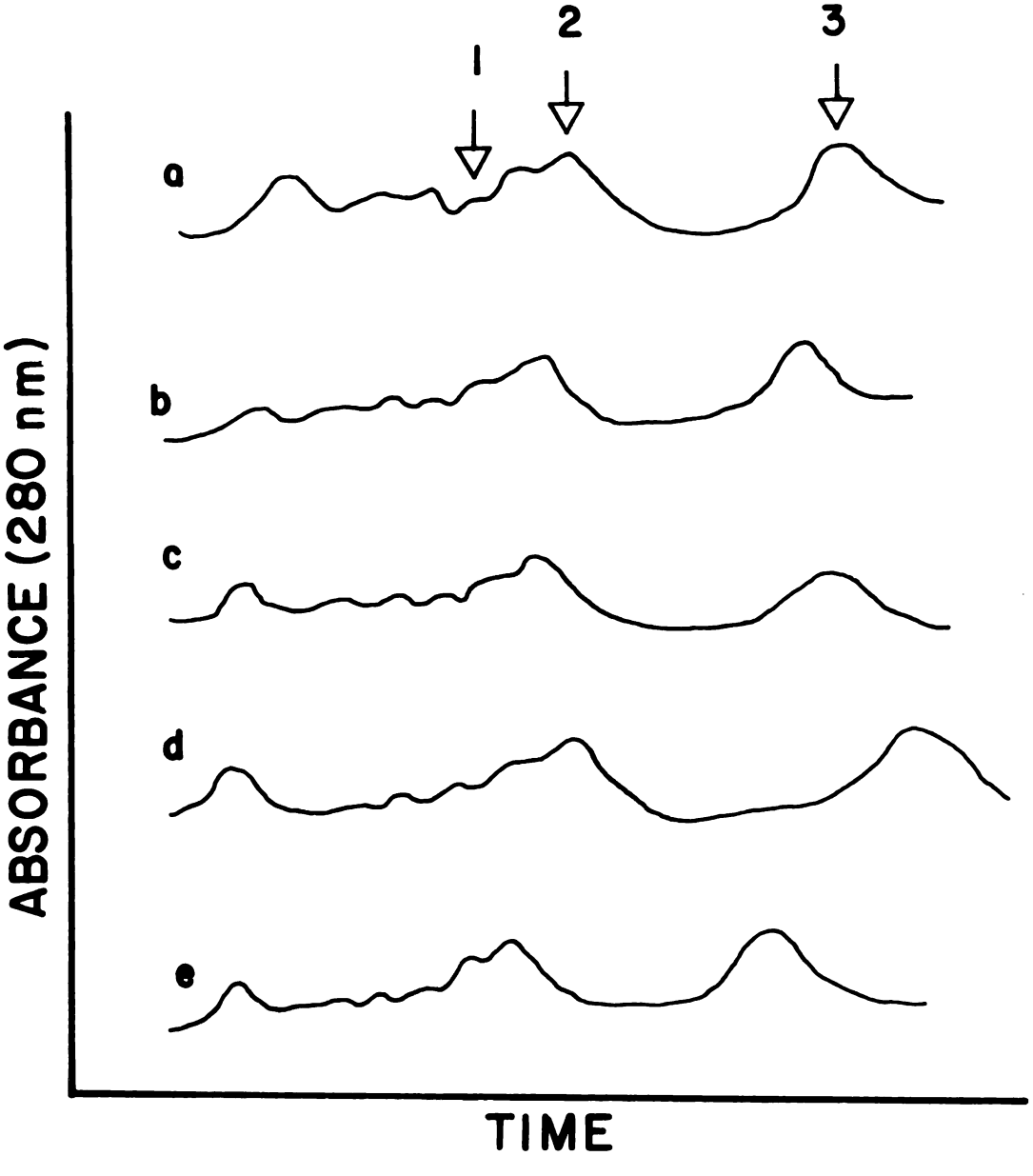

Figure 4

untreated casein are listed in Table 6. Each amino acid, corrected for the enzyme blank, is expressed as g amino acid/16 g N, and also percent of the total amino acid determined by acid hydrolysis. For all five proteins, over 40% of lysine, arginine, sulfur amino acids, leucine, tyrosine, and phenylalanine were each released. This pattern reflects the specificity of the enzymes used. For example, pepsin attacks peptide bond whose carbonyl function is donated by either aromatic amino acids or methionine, while trypsin preferentially hydrolyzes peptide bonds after lysine and arginine residues. The release of the acidic amino acids, aspartic and glutamic acid were minimal, and proline was not liberated. The total amino acids hydrolyzed during digestion were: Untrt, 25.77 g/16 g N; Exunox, 27.52 g/16 g N; Exox, 21.72 g/16 g N; Auto, 29.32 g/16 g N; Auto Glu, 22.50 g/16 g N. Casein exposed to oxidizing oil or autoclaved casein with glucose added appeared from these data to be less susceptible to enzyme digestion. In contrast, the milder treatments, autoclaving or solvent extraction of unoxidized oil, exhibited slight increases in the extent of digestion.

The free essential and nonessential amino acid profiles of the two severely treated proteins, Exox and Auto Glu were significantly altered. The essential amino acids of the Exunox and Auto samples were liberated to

Table 6. Free amino acids in pepsin-pancreatin digest of treated and untreated casein samples

Free Amino Acid	Untrt.[1]		Ex. Unox.[1]		Ex. Ox.[1]		Auto.[1]		Auto. Glu.[1]	
	Digest[2]	(%)[3]	Digest[2]	(%)[3]	Digest[2]	(%)[3]	Digest[2]	(%)[3]	Digest[2]	(%)[3]
Lys	4.03	60.97	4.56	66.57	3.65	51.85	4.75	67.19	2.96	47.90
Thr	0.68	17.66	0.66	17.46	0.42	11.29	0.73	18.77	0.67	17.63
1/2 Cys	0.00	0.00	0.07	17.95	0.06	16.67	0.07	17.95	0.00	0.00
Met	1.27	52.92	1.28	52.67	0.83	37.73	1.50	71.43	1.05	49.76
Val	1.52	21.75	1.44	22.29	1.11	15.39	1.69	22.59	1.26	18.29
Ile	0.79	15.64	0.81	16.63	0.61	12.01	0.95	19.51	0.69	13.99
Leu	4.44	51.21	4.43	54.02	3.73	38.94	4.57	54.47	3.82	44.11
Tyr	2.67	48.11	2.61	50.19	2.25	43.35	2.67	50.76	2.38	45.51
Phe	2.34	49.47	2.28	48.93	1.95	40.79	2.41	50.74	2.11	42.63
Try	1.23	75.93	1.32	74.58	0.83	58.87	1.31	82.39	1.01	52.88
His	0.19[4]	7.85[4]	1.12	46.47	0.76	30.28	1.33	52.16	0.89	33.21
Arg	2.60	80.50	2.46	76.40	2.24	68.29	2.62	80.86	2.24	66.08
Asp	0.23	3.58	0.19	2.99	0.25	3.96	0.23	3.23	0.19	2.98
Ser	1.28	25.96	1.42	28.57	1.06	21.81	1.16	23.63	0.88	17.85

Table 6. Continued

Free Amino Acid	Untrt.[1]		Ex. Unox.[1]		Ex. Ox.[1]		Auto.[1]		Auto. Glu.[1]	
	Digest[2]	(%)[3]	Digest[2]	(%)[3]	Digest[2]	(%)[3]	Digest[2]	(%)[3]	Digest[2]	(%)[3]
Glu	1.40	6.69	1.39	6.84	1.02	4.99	1.63	7.83	1.28	6.13
Pro	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Gly	0.08	5.41	0.1	6.62	0.07	4.83	0.16	10.96	0.07	4.49
Ala	0.26	10.12	0.27	10.59	0.16	6.27	0.30	11.86	0.24	9.30
NH_3	0.40	20.41	0.70	40.23	0.50	26.74	0.86	42.79	0.76	36.36
Met Sulfoxide	0.36		0.41		0.00		0.38		0.00	
Met Sulfone	0.00		0.00		0.22		0.00		0.00	
Total	25.77		27.52		21.72		29.32		22.50	

[1]Untrt., No treatment; Ex. Unox., Casein after extraction of unoxidized safflower oil; Ex. Ox., Casein after extraction of oxidized safflower oil; Auto., Casein autoclaved 5 min 121 C; Auto Glu., Casein and glucose autoclaved 5 min 121 C.

[2]Values expressed as g/16 g N of digest.

[3]Values expressed as percent of total amino acid determined by acid hydrolysis.

[4]Values are low due to technical problems.

approximately the same extent as the Untrt control. However, the release of two nonessential amino acids, glycine and alanine, from the two milder treated proteins was greater than from that of the Untrt casein. All of the liberated essential amino acids of the Exox sample were decreased when compared to those of the Untrt casein. The largest decreases of amino acids were observed for threonine, 36%; valine, 29%; sulfur amino acids, 35%; isoleucine, 23%; and tryptophan, 33%. The concentration of released lysine, leucine, tyrosine, and phenylalanine were reduced by less than 20%. With the exception of aspartic acid, all the nonessential amino acids released from the Exox casein were reduced. Autoclaving the casein-glucose mixture caused a reduction in the release of all essential amino acids, but only in the case of lysine was the reduction greater than that noted for the Exox sample. The hydrolysis of the nonessential amino acids of the Auto Glu sample were also reduced. The release of serine was decreased by 39%; while the liberation of other nonessential amino acids was reduced by less than 20%.

Free Amino Acid Indices of Protein Quality

Various indices have been calculated from the concentrations of essential amino acids liberated by digestion. The Pepsin Pancreatin Digest Index (PPDI;

Akeson and Stahmann, 1964) was calculated (see Appendix for enzymatic digestion of whole egg protein). The PPDI method involves identical digestion of a reference protein, whole egg, and a test protein. The calculation of PPDI includes a comparison of both the essential amino acids released by digestion and those amino acids remaining undigested between the reference and test protein. Correction factors are also included in the calculation to account for differences in the extent of proteolysis between the reference and test protein. Because egg protein has been shown to contain an excessive amount of essential amino acids relative to the amino acid requirement of the rat, Untrt casein was selected as a reference. The PPDI results using the Untrt casein and egg protein as references are shown in Table 7. When egg protein served as the reference, the PPDI of the Untrt, Exunox, and Auto casein were similar; 77, 78, and 76, respectively. However, the more severely treated proteins, Exox and Auto Glu, yielded higher values; 80 and 83, respectively. In contrast, when Untrt casein was taken as the reference, the PPDI varied from 91 for the Exox casein to 100 for the Untrt casein.

An Enzyme Score (Stahmann and Woldegiorgis, 1975) and Enzyme Index were calculated from the essential amino acids released during digestion. The Enzyme Score, which is calculated similarly to the Protein Score, is the

Table 7. Pepsin Pancreatin Digest Index[1] of treated and untreated casein.

Treatments[2]	PPDI	
	Egg Reference	Untrt casein Reference
Untrt	77	100
Exunox	78	96
Exox	80	91
Auto	76	92
Auto Glu	83	94

[1]Akeson and Stahmann (1964).

[2]Untrt, No treatment; Exunox, Casein after extraction of unoxidized safflower oil; Exox, Casein after extraction of oxidized safflower oil; Auto, Casein autoclaved 5 min 121 C; Auto Glu, Casein and glucose autoclaved 5 min 121 C.

smallest ratio of the essential amino acids released from the test protein to that essential amino acid released from the reference protein. The Enzyme Index, derived similarly to the Essential Amino Acid Index, is the geometric mean of all the ratios of essential amino acids released from a test protein to these amino acids released from a reference protein. The Enzyme Score and Enzyme Index for the five proteins are presented in Table 8.

When the released essential amino acids of egg protein were used as the reference, the sulfur amino acids were the limiting amino acids in all proteins. However, when the Untrt casein was used as the reference, the limiting amino acids were; valine for Exunox, threonine for Exox, and lysine for Auto Glu. For the Auto sample, the amino acids released were either the same or greater than those released from the Untrt casein. The Enzyme Score was consistently lower than the Enzyme Index. In addition, the values for the Enzyme Index and Enzyme Score, using egg protein as the reference, were lower than when Untrt casein was the reference protein.

Initial Rate of Digestion: pH Stat Evaluations

The purpose of this experiment was to investigate the effects of treatments on the initial rates of protein

Table 8. Free amino acid composition (expressed as g/16 g N) of pepsin pancreatin digest, and Enzyme Score and Enzyme Index of treated and untreated casein[1]

Amino Acid	Protein Treatment[2]					
	Egg	Untrt	Exunox	Exox	Auto	Auto Glu
Lys	2.70	4.03	4.56	3.65	4.75	2.96
Met, 1/2 Cys	1.90	1.27	1.35	0.89	1.57	1.05
Phe, Tyr	5.80	5.01	4.89	4.20	5.08	4.49
Leus	4.80	4.44	4.43	3.73	4.57	3.82
Ile	0.90	0.79	0.81	0.61	0.95	0.69
Val	1.80	1.52	1.44	1.11	1.69	1.26
Thr	0.60	0.68	0.66	0.42	0.73	0.67
Try	1.10	1.23	1.32	0.83	0.83	1.31
Enzyme Score						
Egg reference		67	71	45	83	55
Untrt casein reference		100	95	62	100	73
Enzyme Index						
Egg reference		89	89	70	94	80
Untrt casein reference		100	99	75	100	85

[1]Amino acid underlined with solid line was used to calculate Enzyme Score when egg protein was the reference. Amino acid underlined with a dashed line was used to calculate the Enzyme Score when Untrt casein was the reference.

[2]Untrt, No treatment; Exunox, Casein after extraction of unoxidized safflower oil; Exox, Casein after extraction of oxidized safflower oil; Auto, Casein autoclaved 5 min 121 C; Auto Glu, Casein and glucose autoclaved 5 min 121 C.

digestion by either pancreatin or trypsin. In addition to determining initial rates of digestion on intact proteins, similar values were also measured on proteins which had been pre-digested with pepsin. The pepsin pretreatment was performed to determine if the protein treatments affected initial pancreatin or trypsin digestion rates after the protein had been partially hydrolyzed.

The initial rates of pancreatin digestion are shown in Table 9. For the intact protein, the initial rate of the Exox casein was significantly lower than the rate for the Untrt or Exunox samples ($P < 0.05$). The initial rates for the autoclaved samples were slightly lower than those of the Untrt control; however, the differences were not significant at the 95% confidence level.

The initial rate results for the pepsin-pretreated proteins were different from those obtained for the intact proteins. The initial rates for the autoclaved samples were significantly lower ($P < 0.05$) than that for the Untrt casein, while the rate for the Exox sample was essentially the same as the Untrt control. The initial rates of digestion for the Exunox, for both the intact and pepsin-pretreated proteins, were slightly greater than those of the Untrt control.

In addition to pancreatin, trypsin was also employed to determine the initial rates of digestion for the five casein samples. The initial rate results

Table 9. Initial rates of pancreatin digestion of treated and untreated casein.

Treatments	Initial Rate (ml base/min.)	
	Intact Protein[1]	Peptic Dig.[1]
Untrt.	0.151 ± 0.001^{ac}	0.138 ± 0.001^{ab}
Ex. Unox.	0.171 ± 0.064^{a}	0.156 ± 0.025^{a}
Ex. Ox.	0.107 ± 0.020^{b}	0.131 ± 0.011^{bc}
Auto.	0.135 ± 0.017^{c}	0.112 ± 0.014^{c}
Auto. Glu.	0.144 ± 0.014^{c}	0.112 ± 0.010^{c}

[1]Mean of 5 Replicates ± SEM.

NOTE: Means not showing common superscript are significantly different ($P < 0.05$).

for tryptic hydrolysis appear in Table 10. For the intact proteins, the initial rates were quite similar except for the rate of the Auto Glu casein which was significantly higher ($P < 0.05$) than the others. The initial rates of the tryptic digestion for the pepsin-pretreated proteins were approximately equal.

Table 10. Initial rate of trypsin digestion of untreated and treated casein.

Treatments	Initial Rate (ml base/min.)	
	Intact Protein[1]	Peptic Dig.[1]
Untrt.	0.156 ± 0.010[ab]	0.157 ± 0.003[a]
Ex. Unox.	0.145 ± 0.006[a]	0.165 ± 0.009[a]
Ex. Ox.	0.137 ± 0.013[a]	0.155 ± 0.055[a]
Auto.	0.141 ± 0.009[a]	0.160 ± 0.000[a]
Auto. Glu.	0.176 ± 0.018[b]	0.150 ± 0.023[a]

[1]Mean of 5 Replicates ± SEM.

NOTE: Means not showing a common superscript are significantly different ($P < 0.05$).

DISCUSSION

Effects of Protein Treatments

Mild Treatments--Autoclaving and Solvent Extraction of Unoxidized Oil

The two milder casein treatments, solvent extraction of unoxidized oil and autoclaving, caused little change from the Untrt casein in the amino acid composition of either the intact proteins or the enzymatic supernatants. In addition, the PER of these two treated proteins was not significantly different from the Untrt sample at the 95% confidence level. Although the gel filtration chromatograms did not indicate any significant differences in the proportion of free amino acids between the treated and untreated proteins, the total in vitro enzymatic release of free amino acids from the Auto and Exunox samples was slightly greater than that of the Untrt casein. This difference in free amino acid release was greater for the Auto sample than for the Exunox sample. Some researchers have suggested that mild treatment increases the susceptibility of proteins to enzymatic digestion because it induces a slight denaturation (Osner and Johnson, 1975). However, since the initial

rate of digestion with either pancreatin or trypsin did not increase after autoclaving, and the rate of digestion monitored by alpha amino nitrogen of Auto casein did not exceed that of the Untrt, the increased release of amino acids might be due to the slight denaturation of casein, caused from autoclaving, which releases some indiginous TCA-soluble material. The increased TCA-soluble alpha amino nitrogen of the Auto Glu sample compared to the Untrt control at zero time of digestion supports this hypothesis.

Casein Exposed to Oxidizing Oil

Amino acid analyses of acid hydrolysates of casein which had been exposed to oxidizing oil demonstrated significant changes from the Untrt casein only with respect to the decreases of sulfur amino acids and tyrosine. Several researchers have found that the major amino acids destroyed after a protein was exposed to oxidizing oil were lysine, arginine, histidine, and methionine (Braddock and Dugan, 1973; Yanagita and Sugano, 1974; Roubal and Tappel, 1967). These authors did not determine tryptophan in the amino acid analyses.

In vitro enzymatic digestion of the Exox casein released a lower amount of amino acids than that released from the Untrt casein. In addition, the protein quality of the Exox sample as measured by PER was significantly lower ($P < 0.05$) than the Untrt casein. The similarity

in the total amino acid content determined after acid hydrolysis of the Exox and Untrt casein, along with the dramatic decrease in amino acids released during enzymatic digestion of the Exox sample compared to the Untrt, suggest that the decrease in protein quality is due to a decrease in amino acid availability.

Probably more than one mechanism was involved in the decrease of availability of the amino acids. As evidenced by the browning that occurred during oxidation of the oil, the reaction of the carbonyl compounds formed during fatty acid oxidation with amino groups of the protein could have been the major mechanism. The basic amino acids were likely the most affected amino acids. Pokorny et al. (1973, 1975) studied the browning reaction that occurs in protein-lipid systems, concluding that various aldehydes, formed from hydroperoxide breakdown, were primary substrates for the nonenzymatic browning. Yanagita et al. (1973) observed a decrease in available lysine which paralleled nonenzymatic browning in a casein-oxidized ethyl linoleate mixture. Thus, this reaction might account for some of the decreases observed in available lysine and arginine.

As fats undergo oxidation in a protein-lipid system, the sulfur amino acids are concomitantly oxidized (Tannenbaum et al., 1969). The presence of methionine sulfone in the enzymatically released amino acids

suggests that the methionine was oxidized in the Exox casein. The products of sulfur amino acid oxidation do not have the same nutritive potency as methionine and cystine. For example, methionine sulfoxide has only 60% of the biological value of methionine; while methionine sulfone and cysteic acid have no biological activity (Andrews et al., 1976). Cuq et al. (1973) observed that methionine sulfoxide and sulfone were not enzymatically released from casein after treatment with H_2O_2, indicating that the peptide bonds involving oxidation products of methionine are resistant to enzymatic hydrolysis.

It was suggested that the free radicals produced during lipid oxidation react with proteins (Karel, 1973). Certain aromatic amino acids, such as tryptophan and histidine, have been demonstrated to possess antioxidative properties (Mitsuda, 1967). It was suggested that amino acids act as antioxidants by reacting with peroxide radicals to form inactive peroxyanions. Such free radical attack of reactive aromatic amino acid residues could therefore induce losses of protein nutritional quality. This reaction could possible explain the 12% decrease of tryptophan in the acid hydrolysate of the Exox sample.

In addition to a reduction in the enzymatic release of amino acids, the overall rate of digestion as monitored by the alpha amino nitrogen content of the TCA supernatants of Exox casein was slower than that of

the Untrt sample. A decreased initial rate of pancreatin digestion of the intact Exox casein was also noted. These results are in agreement with those of Yanagita et al. (1973) who demonstrated a decrease in in vitro digestibility with pepsin and trypsin when casein and ethyl linoleate were incubated until browning occurred. The decrease in digestion rate could be due to the modification of side chains of amino acid residues normally susceptible to enzymatic cleavage, such as those involving carbonyl compounds reacting with amino groups. The increased TBA values of Exox sample indicate that the dialdehyde compound, malonaldehyde, was present after the oxidized oil was removed. In addition as previously discussed, the oxidation products of sulfur amino acids might contribute to the inhibition of enzymatic hydrolysis. Therefore, the combination of blocked amino acids by carbonyl compounds and the presence of oxidation products of the sulfur amino acids could explain partially the reduced digestion rate.

Casein Autoclaved with Glucose

Although the PER of the Auto Glu casein was not significantly lower than that of the Untrt casein ($P < 0.05$), the amino acid profile of the acid and enzymatic hydrolysates was altered. The lysine and sulfur amino acids of the acid hydrolysate of Auto Glu casein were lower than those of the Untrt hydrolysate. All other

amino acids in the acid hydrolysate of the Auto Glu appeared to be unaffected by the treatment. However, all the amino acids released from the Auto Glu casein during enzymatic digestion were reduced relative to those liberated from the Untrt sample. Autoclaving a casein-glucose mixture results in a series of reactions known as nonenzymatic browning or the Maillard Reaction. These reactions have been studied extensively (Adrian, 1974; Bender, 1972; Feeney et al., 1975). These authors have found that the basic amino acids, the sulfur amino acids, and tryptophan were the most reactive amino acids in the Maillard Reaction. Rao and McLaughlin (1967) found that in a casein-glucose mixture, the available lysine decreased faster than the available methionine. These results support the data of the present study. The lysine released after in vitro digestion was reduced 27%, while the sulfur amino acids were reduced 16%.

The total amount of amino acids released from Auto Glu casein during in vitro enzymatic digestion was less than that released from the Untrt sample; 22.50 g/16 g N versus 25.77 g/16 g N. The other experiments which examined the rate and extent of hydrolysis during enzymatic digestion were not sufficiently sensitive to detect the difference in the amount of amino acids released. The extent of hydrolysis of the Auto Glu was not significantly different from that of Untrt

casein at the 95% confidence level. When the initial rates of digestion were investigated, only in the case of the pancreatin initial rate of the pepsin-pretreated Auto Glu sample was there a significant decrease ($P < 0.05$) from the rate of the Untrt casein. Therefore, only by examining the released amino acids could this difference be detected.

Protein Quality Indices

No significant differences were observed in the Sephadex G-25 chromatograms of the TCA-soluble material released by in vitro enzymatic digestion of the test proteins. Therefore, the molecular size distribution of the digestion products could not be employed as a qualitative indicator of protein quality.

Although slight differences between the initial rates of the treated and untreated samples were observed, the most sensitive indicator of protein quality was derived from analysis of the amino acids released during in vitro digestion. To quantitate the differences in nutritional quality among the proteins, various quality indices involving the essential amino acids liberated during in vitro enzymatic digestion were used. A comparison of these indices with PER results is presented in Table 11. Linear correlation coefficients were calculated to provide a criterion for comparison of the

Table 11. Comparison of PER results with Protein Score, Essential Amino Acid Index (EAAI), and in vitro digestion indices for treated and untreated casein.

Treatments	Protein Quality Indices									
	PER	Rel. PER	Pro.[1] Score	EAAI[2]	PPDI[3]		Enzyme Score[4]		Enzyme Index[5]	
					Egg	Untrt.	Egg	Untrt.	Egg	Untrt.
Untrt.	3.07	100	79	97	77	100	67	100	89	100
Ex. Unox.	3.14	102	81	97	78	96	71	95	89	99
Ex. Ox.	2.30	75	83	95	80	91	45	62	70	75
Auto.	2.83	92	71	95	76	92	83	100	94	100
Auto. Glu.	2.78	91	71	95	83	94	55	73	80	85
Correlation Coefficient			0.66	0.77	-0.42	0.78	0.69	0.85	0.83	0.90

[1]Protein Score (FAO/WHO, 1973).

[2]Essential Amino Acid Index calculated using provisional pattern of FAO/WHO 1973.

[3]Pepsin-Pancreatin Digest Index using either egg protein or untreated casein as the reference (Akeson and Stahmann, 1964).

[4]Enzyme Score (Stahmann and Woldegiorgis, 1975) using either egg protein or untreated casein as the reference.

[5]Either egg protein of untreated casein was used as the reference.

correlation of these in vitro indices of nutritional quality with bioassay results (PER).

The Protein Score and Essential Amino Acid Index did not reflect the nutritional quality in the treated proteins as determined by the PER method. Because the Protein Score and Essential Amino Acid Index are based on the total rather than the biologically available amino acids, these methods are not accurate indicators of the nutritional quality of the proteins.

The results demonstrate that, in proteins in which amino acid availability is the predominant limiting factor in the nutritional quality of the protein, an in vitro enzymatic digestion provides a better indication of the protein quality than the total amino acid indices. The PPD Index (Akeson and Stahmann, 1964), which utilizes amino acid data from an in vitro digestion procedure, was calculated for the five casein samples. For the two most severely treated proteins, Exox and Auto Glu, the PPD Index was observed to be greater than that of the Untrt casein, contrary to results obtained by the PER method. These results were supported by those of Stahmann and Woldegiorgis (1975), demonstrating that the PPD Index overestimates the quality of moderately damaged proteins. Since egg protein contains an excess of certain amino acids relative to the amino acid requirement of the rat, Untrt casein was tested as a

reference protein for the in vitro nutritional quality estimates. Even when Untrt casein was the reference, the PPD Index overestimated the protein quality of Exox and Auto Glu samples.

The method of calculation employed in the PPD Index determination presumably is responsible for the overestimation of protein quality. The major problem in the PPD Index calculation concerns the use of a factor which corrects for differences in the extent of proteolysis during in vitro digestion between the test and reference protein. Using this correction factor, one assumes that the amino acids of the test protein are released to the same extent as those of the reference protein. In the case of the Exox and Auto Glu samples, which have undergone chemical modification, some of the bonds which are normally susceptible to digestive enzymes are resistant to enzymatic attack. Therefore, because the amount of amino acids released from these moderately processed proteins will not be equal to the amount released from the reference protein, the use of the correction factor is not valid. A reduction in the PPD Index is only observed when there is a large decrease in the initial release of an amino acid, such as in the case of severely processed samples.

Another index of protein quality, Enzyme Score (Stahmann and Woldegiorgis, 1975), which is based only

on those essential amino acids released during in vitro digestion, was tested as an alternate indication of protein quality. By using a reference of amino acids released from either egg protein or Untrt casein, this evaluation indicated that protein quality was lower than the relative protein quality as determined by bioassay (PER). However, the Untrt casein reference did provide a better correlation with PER than was observed with the egg protein reference.

A limiting feature of the Enzyme Score is the fact that the concentration of the most limiting released amino acid used for calculation might be lower than that found if digestion were complete. In the Exox casein, the initial rate and overall digestion were lower than those of the Untrt sample. Therefore, at the end of the 27 h digestion period, fewer amino acids were released from the Exox. However, in vivo, the length of the intestine allows for more of the slower digested material to be absorbed (Rerat, 1976). Thus, the value for the limiting amino acid after 27 h of digestion is probably lower than it would have been if digestion were complete.

Another expression of protein quality, Enzyme Index, was calculated by computing the geometric mean of all the ratios of the released essential amino acids of the test protein to those released in the reference protein. By taking a weighted mean of the ratios, instead

of using only the smallest ratio as in the Enzyme Score, potential biases associated with incomplete digestion were minimized. The values for the Enzyme Index, using either the Untrt casein or egg protein as the reference, gave the best correlation to the PER results of the treated proteins.

CONCLUSION

The difference in the nutritional quality of the four treated caseins was reflected in their varying PERs. Various aspects of an in vitro enzymatic digestion were evaluated as indices of protein quality by comparing them with the PER results for the casein samples. Although slight differences in the rates of the in vitro digestion were observed, the best indicator of protein quality was the profile of amino acids released during pepsin-pancreatin sequential digestion. Various indices using the essential amino acids released during digestion were calculated. When the untreated casein was used as the reference protein, Enzyme Score and Enzyme Index correlated best with the bioassay results.

Although further investigation is needed to verify the correlation between the Enzyme Score or Enzyme Index and PER, the in vitro enzymatic digestion appears to be a useful method for protein quality determination. The in vitro digestion procedure is relatively rapid and provides information concerning amino acid availability.

APPENDIX

APPENDIX

Preparation of Ninhydrin Solution

1. 0.2 M citrate buffer (pH 5.0) was prepared using 4.3 g citic acid and 8.7 g Na_3citrate·$2H_2O$ in 250 ml water.
2. To the above solution 400 mg of reagent grade $SnCl_2$·2 H_2O was added.
3. 250 ml of methyl cellusolve containing 10 g dissolved ninhydrin was added to the citrate buffer containing the $SnCl_2$. This solution was aged two days before using and was prepared fresh every 7-8 days.

Reagents for the TBA Test

Aldehyde-free redistilled absolute ethanol--was prepared by dissolving 1.5 g $AgNO_3$ in 3 ml distilled water and mixing with 1 l of absolute ethanol. Three grams KOH were dissolved in 15 ml of warm absolute ethanol. When cool, the two solutions were mixed thoroughly. The resulting precipitate was allowed to settle, then filtered. The filtrate was distilled and stored in an amber glass container.

TBA reagent--was prepared by transferring 1.4 g TBA (Eastman Organic Chemicals) into a volumetric flask and diluting to 100 ml with aldehyde-free redistilled absolute ethanol. The mixture was sonicated 1 h, and then filtered through Whatman no. 41 filter paper. The reagent was prepared immediately before use.

Moisture and Nitrogen Content of Casein Samples Used in PER Diets

Casein Treatment	Percentage Nitrogen (%)	Moisture (%)
No treatment	14.5	7.11
Casein after exposure to unoxidized oil	14.3	10.00
Casein after exposure to oxidized oil	12.4	6.97
Autoclaved 5 min. 121 C	15.1	4.84
Casein glucose mixture autoclaved 5 min. 121 C	8.24	5.36

Enzymatic Digestion of the Dried Whole Egg Solid Sample[1]

	Total[2]	Digest[3]
Lys	7.1	2.7
His	2.8	0.8
Arg	6.6	3.1
Asp	9.8	0.2
Thr	4.6	0.6[4]
Ser	7.4	0.5[4]
Glu	12.6	0.5

(continued)	Total[2]	Digest[3]
Pro	4.7	0.00
Gly	3.1	2.8
Ala	5.5	5.0
1/2 Cys	2.3	1.9
Val	6.1	6.8
Met	3.3	2.4
Ile	8.2	9.0
Leu	8.2	9.0
Tyr	4.0	4.1
Phe	4.8	5.3
Tyr	1.9	1.9
Total	97.4	25.2

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[1]Values are from Dimler (1975).

[2]Values are expressed as g amino acid residue/16 g N of protein.

[3]Values are expressed as g amino acid/16 g N of digest.

[4]Values are estimated from Dimler (1975).

Calculation of In Vitro Digestion Indices

1. Pepsin-Pancreatin-Digest-Index

   A. The total essential amino acids of the egg and test protein are listed along with the essential amino acids which were released during digestion (digest) and those remaining undigested (residue).

   B. The quantity of each digested amino acid was expressed as a percentage of the total digested amino acids for both proteins. The same procedure was followed for the residue amino acids for both proteins.

   C. Ratios of each amino acid of the test protein to the egg protein expressed as a percentage were calculated for both the digest and residue fractions. These ratios are referred to as egg ratios.

   D. Geometric means for both egg ratio fractions were calculated. These mean values were then corrected for the degree of proteolysis of the test protein relative to the egg protein. These corrected means are weighted in accordance with the percentage each represents of the total egg protein, and averaged geometrically to obtain PPDI.

2. Enzyme Score

Enzyme score is calculated like the Protein Score. It is the smallest ratio of the essential amino acid released during digestion of the test protein to that amino acid released from the reference protein.

3. Enzyme Index

Enzyme Index is the geometric mean of the ratios of the essential amino acids released during digestion of the test protein to those amino acids released from the reference protein.

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