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# Evaluation of heat processing on soy proteins using a pH stat to monitor tryptic hydrolysis

by

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# INTRODUCTION

Heat processing influences the nutritional value, organoleptic qualities, enzyme activity, protein solubility and functionality of soy protein products. The most common methods for analyzing the heat treatment of soy products are the protein dispersibility index (PDI) and the nitrogen solubility index (NSI). These methods are based on the concept that heat decreases protein solubility. Measurement of the susceptibility of soy proteins to tryptic hydrolysis may be a less time consuming method for evaluating heat treatment. Soy protein becomes more susceptible to tryptic hydrolysis as the degree of heat treatment increases, due to denaturation of the protein and inactivation of the trypsin inhibitors. Therefore, the rate of hydrolysis would reflect the kind of heat treatment the soy protein had received.

The objective of this research is to evaluate the effects of heat processing on soy proteins, using a pH stat to monitor tryptic hydrolysis. The pH stat measures protein hydrolysis as a function of time by automatically titrating the hydrogen ions produced, as the reaction proceeds at a constant pH. The initial rate of base addition can be used as a measure of proteolysis.

I evaluated three soy flours that had received different heat treatments. The effects of substrate concentration, enzyme concentration, trypsin inhibitors and instrumental parameters on the rates of proteolysis were investigated.

# LITERATURE REVIEW

## Effect of Heat on Soy Proteins

### Protein solubility

Early studies reported that moist heat treatment of soy proteins resulted in decreased solubility (Beckel et al., 1942; Belter and Smith, 1952). The protein solubility of defatted meal decreased from an initial 80% to 20-25% after 10 minutes of steam heating at 100°C (Belter and Smith, 1952). Little difference was observed in the rate of insolubilization of protein in defatted or full-fat soybean flakes.

A number of terms have been used to designate the solubility of soy protein. The two most frequently encountered terms are nitrogen solubility index (NSI) and protein dispersibility index (PDI). Both methods involve extraction of the protein with water followed by Kjeldahl analysis of the extracts. The main difference in the two methods is in the extraction procedure. In the NSI or slow stir method, the extraction is made by slowly stirring for 2 hours. In the PDI or fast stir method, the sample is extracted for 10 minutes using a high speed mixer equipped with cutting blades. PDI values are generally higher than NSI values, apparently because of the greater shearing action during the extraction procedure (Wolf and Cowan, 1971).

NSI or PDI is often used to determine the amount of heat treatment soy products have received. The processing variables that have the most influence

on protein solubility and protein denaturation are time, temperature and moisture content (Becker, 1971). An increase in any of these variables results in decreased protein solubility. However, the rate of denaturation is very slow below a threshold value characteristic of each variable (Becker, 1971).

The desolventizing step is the most critical in determining protein denaturation during soybean processing (Belter and Smith, 1952). Only a small decrease in protein solubility occurs during cracking, dehulling, conditioning, flaking and extracting. To retain high protein solubility, a flash desolventizer system is usually used. In the flash desolventizer system, superheated solvent vapors simultaneously desolventize and convey the flakes to a cyclone separator where the flakes and vapors are separated. The flakes are further desolventized in a meal deodorizer by an inert sparge gas that flows counter to the meal. A white flake is obtained with PDI values of 70-90 (Becker, 1971).

A desolventizer toaster is extensively used to produce toasted meal for ruminant and poultry feeds. The desolventizer toaster consists of a vertical vessel containing steam heated tray sections. Hexane wet flakes are sparged with steam in the upper trays. Steam condenses in the flakes vaporizing the hexane and raising the moisture content to about 20%. The flakes are heated to about 107°C in the lower trays, which reduces the

moisture content by several per cent. PDI values of toasted meal range from 10-30 (Becker, 1971).

Fukushima (1959a) investigated the correlation between water dispersible nitrogen of heat treated soy flour and the degree of protein denaturation. The water dispersible nitrogen rapidly reached a minimum and then increased again when equal weights of flour and water were heated at 110° or 120° C. As the autoclaving temperature or water content of the flour increased, an increase in dispersible nitrogen occurred beyond the minimum point (Fukushima, 1959a). At this point the protein was denatured to a soluble form.

Denaturation was measured by the change in susceptibility of the water extractable proteins of soy flour to Aspergillus sojae protease before and after heat treatment. The difference is proportional to the amount of native protein in the sample. Hydrolysis was carried out at pH 7.2 for 30 minutes. The degree of hydrolysis was determined by the absorbance of the trichloroacetic acid (TCA) soluble fraction at 660 nm after reaction with Folin's reagent. Denaturation increased with temperature and moisture content. The native protein in the water extractable proteins of heated soy flour disappeared at the minimum point of nitrogen dispersibility. Water dispersible nitrogen correlated with the amount of native protein only within the range where the minimum water dispersible nitrogen was obtained.

Fukushima (1959b) developed a method that was quantitative and could be applied to soy flours with various heat treatments. The method is based

on the stability of native proteins to proteolysis and their susceptibility to digestion after denaturation. Native protein was determined by the quantity of protein that is solubilized but remains unhydrolyzed after a 20 hour digest (30° C, pH 7.2) by Aspergillus sojae protease. A linear relationship exists between the optical density of the protein solution plus TCA at 660 nm and the native protein present in soy flour.

Protein aggregation

Changes in protein solubility are a measure of the extent of denaturation, but little information is obtained on the nature of the changes occurring at the molecular level. Several studies have been conducted using dilute solutions of water extractable soy proteins or isolated components to investigate some of these changes. Watanabe and Nakayama (1962) followed the changes occurring in the ultracentrifugal pattern of dilute solutions of water extracts of defatted soy meal (8.4 mg/ml). The ultracentrifugal analysis indicated that the 11 S and 15 S fractions plus part of the 7 S fraction aggregated when heated at 80° C or higher. Similar results were obtained using gel filtration (Saio et al., 1968).

Catsimpoolas et al. (1969) reported that solutions of soybean 11 S protein (3.4 mg/ml) became turbid when heated above 70° C and formed a precipitate at 90° C. Heating dissociated the 11 S protein into subunits as indicated by disc electrophoresis, although some undissociated 11 S remained. Heating at

90°C for 1 hour did not destroy the ability of the 11 S protein to react with its antibody. Using changes in turbidity, Catsimpoolas et al. (1970) reported that the rate and extent of aggregation of the 11 S protein were increased by low ionic strength and by mercaptoethanol. The maximum rate of aggregation was observed between pH 4 and 6.

Wolf and Tamura (1969) followed the changes in ultracentrifugal composition of soybean 11 S protein (5 mg/ml) heated to 100°C. The 11 S component disappeared and was replaced by a soluble aggregate and a 3-4 S fraction. The soluble aggregate increased in size and precipitated on continued heating. Only the 3-4 S fraction was left in solution. Precipitation occurred more rapidly when 0.01 M mercaptoethanol was added. However, no soluble aggregate was detected. A soluble aggregate but no precipitation occurred when a sulfhydryl-blocking agent (N-ethylmaleimide) was added. Wolf and Tamura (1969) concluded that heating disrupts the quaternary structure of the 11 S protein and separates the subunits into a soluble and insoluble fraction. The precipitation reaction was proposed to involve hydrophobic interactions that are promoted by cleaving the disulfide bonds in the molecule. Watanabe and Nakayama (1962) did not observe the 3-4 S fraction when the water extractable soy proteins of defatted meal were heated, suggesting that additional reactions may occur when the 11 S protein is heated in the presence of other soy proteins.

Cumming et al. (1973) correlated the effects of heat on isolated solutions of soy protein to reactions occurring during thermoplastic extrusion. Using disc gel and sodium dodecyl sulfate electrophoresis, they found that defatted soy meal separated into subunits and/or became insoluble during thermoplastic extrusion.

Tombs (1967) reported that approximately 70% of the protein in soybeans is present in protein bodies, ranging from 2 to 20 $\mu$ in diameter. Johnson and Snyder (1978) investigated the effect of heat on soy protein during soymilk preparation. They observed a number of protein bodies in soy slurries that were heated prior to grinding. However, the protein bodies were not seen in soy slurries that were not heated before or during grinding. Heating the soybeans before grinding seemed to heat fix the protein bodies, keeping them intact when the soybean cells were disrupted. The heat fixed protein could be redispersed by homogenization (Johnson and Snyder, 1978).

## Gel formation

Circle et al. (1964) discussed the importance of the rheological properties of soy protein isolates in aqueous dispersions with respect to their uses in food systems. Soy proteinates in aqueous dispersions display many rheological properties including the ability to form a gel structure, that could act as a matrix for holding moisture, lipid, polysaccharides or

other ingredients in a food product. The gelation of aqueous dispersions of soy proteinates is dependent on temperature, pH, time of heating and protein concentration (Circle et al., 1964). Circle et al. (1964) reported an increase in viscosity followed by gelation, when commercial soy proteinates were heated at concentrations above 7%. Catsimpoolas and Meyer (1970) proposed a sol-progel-gel transition for heat gelation of acid precipitated globulins. Heating concentrations of soy globulins greater than 8% converts them into a progel state that gels on cooling. The gel is reversibly converted to the progel by reheating. Excessive heating or addition of disulfide cleaving agents results in a metasol state that does not gel on cooling, indicating that disulfide bonds are an important factor in heat gelation.

Enzyme activity

Lipoxygenase catalyzes the oxidation of unsaturated fatty acids that can produce off flavors in soy products. Baker and Mustakas (1973) reported that lipoxygenase is completely inactivated by 15 minutes at 85° C during immersion cooking of soybeans. Lipoxygenase is more heat labile than urease or the soybean trypsin inhibitors. Both urease and soybean trypsin inhibitor activity are destroyed at about the same rate under similar cooking conditions (Albrecht et al., 1966). However, Borchers et al. (1947) pointed out that urease is more sensitive to heat inactivation than the soybean trypsin inhibitors. Baker and Mustakas (1973) found that the inactivation of the

trypsin inhibitors is more time dependent than urease. They pointed out that the rates of inactivation of urease and the trypsin inhibitors are about the same if the heat treatment is sufficiently long. However, if a high temperature, short time process is used, trypsin inhibitor activity may still be present after urease is completely inactivated. One hour at 74° C or 15 minutes at 100° C is sufficient to totally inactivate lipoxygenase, urease and the trypsin inhibitors (Baker and Mustakas, 1973).

The most widely used methods for measuring the extent of heat treatment of soy proteins are protein solubility and urease activity. Measurement of urease activity in toasted soy meal is the official method used in the soybean processing industry. This method measures a change in pH for a given reaction time of a weakly buffered system of soybean meal and urea (Caskey and Knapp, 1944; Bird et al., 1947). A meal that gives a pH increase of 0.02 to 0.25 at 30° C is usually accepted by soybean processors as properly toasted for good nutrition (Croston et al., 1955). Caskey and Knapp (1944) correlated the urease assay of several soy meals that received different heat treatments with chick feeding studies. The meals that gave the poorest growth response exhibited the highest urease activity. Bird et al. (1947) also found that the urease assay was acceptable in detecting inadequately heated meals, in a study comparing urease values with the growth response of chicks.

Nutritional value

The early work of Osborne and Mendel (1917) established the nutritional superiority of properly heat processed soybeans over raw soybeans. They reported that soybeans would not support the growth of rats unless cooked in a steam bath. A vast amount of literature that confirmed and extended this observation to many other species has been reviewed (Liener, 1958). In general, the factors affecting the beneficial effects of heat treatment on the nutritive value of soybeans are temperature, duration of heating and moisture. Under laboratory conditions, maximum nutritive value of soybean protein is achieved by treatment with live steam for about 30 minutes or by autoclaving at 15 pounds pressure for 15-20 minutes (Klose et al., 1948; Borchers et al., 1948; Smith et al., 1964; Rackis, 1965, 1966). The nutritive value of soybean meal is decreased by either under- or over-heating. Protein efficiency ratio (PER) is widely used for biologically evaluating protein quality. PER is defined as the weight gain of a growing animal divided by its protein intake. PER values range from 0.65 for raw to 1.96 for optimally heated, and down to 1.78 for overheated meals in commercial processing (Simon and Melnick, 1950).

The beneficial effects of heat treatment on soy protein are not completely understood. Rackis (1974) stated that raw full-fat and defatted soy flours inhibit growth, depress metabolizable energy and fat absorption, reduce protein digestibility, cause pancreatic hypertrophy, stimulate hypersecretion of pancreatic

enzymes and reduce amino acid, vitamin and mineral availability. Several reviews on the antinutritional factors present in soybeans have been published (Liener and Kakade, 1969; Rackis, 1974; Liener, 1976). Protease inhibitors have been generally believed to be largely responsible for the antinutritional properties of raw soy products. An increase in the nutritive value of soybean meal and a decrease in pancreatic hypertrophy occur as trypsin inhibitors are destroyed (Rackis, 1972). Only about 15 minutes of steaming at 100°C is required to achieve maximum protein efficiency and to inactivate the trypsin inhibitors in full-fat or defatted flakes (Rackis, 1966). Trypsin inhibitors can be inactivated in soaked, whole soybeans by boiling for 5 minutes (Albrecht et al., 1966).

Growth rates of rats fed raw soybean meal supplemented with tyrosine, methionine, threonine and valine were comparable to those fed toasted meal diets; however, pancreatic hypertrophy still occurred (Booth et al., 1960; Khayambashi and Lyman, 1966). When toasted meal diets were supplemented with amino acids, particularly methionine, a further increase was observed in growth rates and protein efficiency.

Liener et al. (1949) reported that adding a partially purified preparation of trypsin inhibitors to autoclaved meal, so as to provide the same level of inhibitory activity as the raw soybeans, did not reduce the PER to that of raw soybeans. This effect was observed both in the presence and absence of

supplemental methionine. Rackis (1974) stated that maximum PER values of soy protein were reached when only 79% of the trypsin inhibitors was inactivated. When 50-60% of the trypsin inhibitors was inactivated, pancreatic hypertrophy was no longer present. Kakade et al. (1972) found no correlation between trypsin inhibitor activity and PER, but they found a negative correlation between the weight of the pancreas and PER.

Kakade et al. (1973) evaluated the effects of trypsin inhibitors in crude extracts of soybeans. They removed the trypsin inhibitors from the extracts by affinity chromatography and compared these extracts with unheated and heated controls. They reported that approximately 40% of the difference in PER and in vitro digestibility between the raw and heated soybean extract, as well as 40% of the pancreatic hypertrophy produced by the unheated soybean extract, could be attributed to the trypsin inhibitors. Kakade et al. (1973) concluded that a combination of the effects of the trypsin inhibitors and the refractory nature of the undigested protein were the principal causes of growth inhibition in raw soybeans. They postulated that a common mechanism may be involved that leads to hypersecretion of the pancreas. Both undigested protein and trypsin inhibitors reduce the effective level of intestinal trypsin, thus counteracting the feedback inhibition of pancreatic secretion normally exerted by this enzyme. Sulfur-containing amino acids are diverted from synthesis of body tissues to synthesis of pancreatic enzymes, that are rich in these amino acids. This serves to accentuate

the already critical situation, since methionine is the limiting amino acid in soybeans.

Hemagglutinins have also been suggested as exerting an antinutritional effect in legumes (Liener, 1974). Liener (1953) reported that the growth of rats was significantly retarded when purified soybean hemagglutinin was added to a diet containing heated soybean meal. However, little growth inhibition was observed when the food intake of the control group was restricted to the food intake of the group fed the hemagglutinin. Birk and Gertler (1961) reported poor correlation between hemagglutinin activity and growth inhibition of several soybean fractions. Turner and Liener (1975) studied the effects of hemaggultinin on the PER and pancreatic hypertrophy of rats. Soybean hemagglutinin was removed from a crude extract of unheated soy flour by affinity chromatography. Turner and Liener (1975) compared the growth of rats fed a diet containing the hemagglutinin free extract with the growth of rats fed the unheated extract. Removal of the hemagglutinin from the soy flour extract had little effect on the PER or pancreatic hypertrophy of rats. These studies indicated that soybean hemagglutinin plays a minor role in the deleterious effects of unheated soy flour.

Several other antinutritional factors that are eliminated by heat have been found in soybeans. Phytic acid decreases the availability of several essential minerals, including zinc, magnesium, copper, molybdenum, calcium, magnesium and iron (Rackis, 1974). Heat or chelating agents can either eliminate the binding properties of phytate or facilitate in the absorption of the minerals.

The goiterogenic effect of soybeans can be effectively abolished by heat treatment or by addition of a small amount of iodine (Block et al., 1961). Heat processing also seems to increase the availability of some vitamins (Rackis, 1974).

Excessive heat treatment may decrease the nutritive value of soy proteins by destroying cystine and lysine (Clandinin et al., 1947; Evans et al., 1951; Taira et al., 1969). Cystine is very heat sensitive. As much as 1/2 - 2/3 of the cystine content of soybean protein may be destroyed by excessive heat treatment (Evans et al., 1951; Taira et al., 1969). When soybean protein is overheated, lysine may be destroyed or made physiologically unavailable (Block et al., 1946). The $\epsilon$-amino group of lysine may interact with reducing groups of sugars. These bonds are not susceptible to tryptic cleavage and lead to a decrease in soy protein digestibility.

Numerous attempts have been made to develop relatively simple and rapid in vitro techniques for predicting the nutritive value of proteins without resorting to expensive and time-consuming experiments. Since the nutritive value of soybeans is markedly influenced by processing conditions, in vitro techniques would be particularly desirable.

Two main factors determine the nutritional value of a protein: 1) the amino acid profile and 2) the digestibility and availability of the protein. Protein digestibility has been measured by several in vitro procedures. Akeson and Stahmann (1964) reported that pepsin-pancreatin digest values for twelve proteins

were highly correlated with biological values from the literature. This index was calculated from amino acids released by an in vitro digestion with pepsin followed by pancreatin. Buchanan and Byers (1969) reported satisfactory agreement between rat bioassays and total digestible nitrogen measured by a papain digestion of wheat leaf protein concentrate. Total digestible nitrogen was calculated from the difference between the substrate nitrogen and the undigested nitrogen, expressed as a percentage of the substrate nitrogen. Saunders et al. (1973) indicated that there was favorable agreement between the pepsin-pancreatin digest and in vivo data. However, they reported poor correlation between the papain digestion assay and in vivo data. A pepsin-trypsin method measuring total digestible nitrogen correlated well with in vivo data (Saunders et al., 1973).

The extent of enzymatic hydrolysis may be followed by measuring the increase in free amino groups, determined by the 2, 4, 6-trinitrobenzenesulfonic acid method (Fields, 1972). Maga et al. (1973) reported that initial rates of hydrolysis by trypsin were good indicators of the digestibility of some common protein sources. A pH decrease in the reaction mixture was used to follow proteolysis. During the hydrolysis of peptide bonds, hydrogen ions are released from newly formed amino groups, causing a decrease in pH. An equilibrium exists between $-NH_3^+ \rightleftharpoons -NH_2 + H^+$, depending on the pH of the reaction mixture and the pK values of the amino groups. The number of hydrogen ions produced is proportional to the number of peptide bonds split. Using a modification of Maga's procedure, Vavak (1975) investigated the correlation between several

enzyme systems and in vivo digestibility data. Good correlation was reported using trypsin, pepsin-trypsin, trypsin-chymotrypsin or trypsin-chymotrypsin-peptidase combinations. Hsu et al. (1977) reported that the pH decrease after 10 minutes digestion with a trypsin-chymotrypsin-peptidase system, modified from Vavak's procedure, had the highest correlation with in vivo digestion. They reported that their method was sensitive enough to detect the effects of processing and the presence of protease inhibitors. Hsu et al. (1977) pointed out that substances with strong buffering capacities may affect the results. However, in the common food systems they investigated, little effect on in vitro digestibility was observed due to buffering.

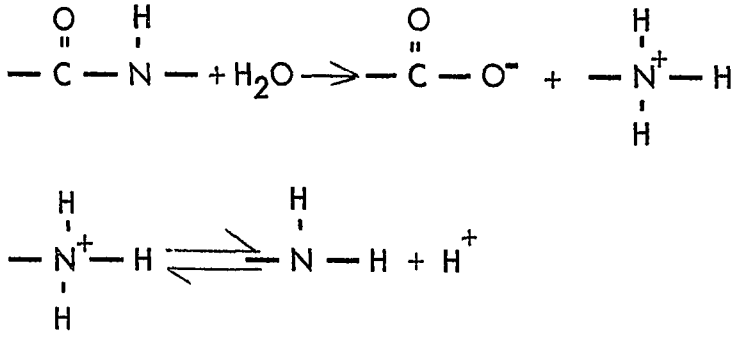
## Hydrolysis of Peptide Bonds

### General reaction

Proteins are made up of amino acids linked through their carboxyl and $\alpha$-amino groups, forming peptide bonds. A large number of enzymes catalyze the hydrolysis of proteins whereby peptides or amino acids are liberated.

Proteolytic enzymes may be divided into two groups, endopeptidases and exopeptidases. Endopeptidases attack interior as well as terminal peptide bonds of polypeptide chains. They are also referred to as proteases. Examples of this group of enzymes are pepsin, elastase, trypsin and chymotrypsin. Exopeptidases are enzymes which attack only the terminal peptide bonds of peptide chains. Carboxypeptidase and aminopeptidase are examples of exopeptidases.

The hydrolysis of a peptide bond by a proteolytic enzyme at an alkaline pH can be represented by the following equations:





At an alkaline pH the carboxyl group is completely ionized, since its ionization constant is approximately 4 in a polypeptide chain (Tanford, 1962; Mihalyi, 1978). The pK of an amino group in an amino acid is around 9, but it is lowered to about 8 when the group exist as an N-terminal group (Tanford, 1962).

## Tryptic hydrolysis

Trypsin possesses a very narrow substrate specificity. It catalyzes the hydrolysis of bonds involving the carboxyl group of arginine or lysine (Neurath and Schwert, 1950). The general formula $R-\overset{O}{\overset{\|}{C}}-X$ can be used to describe trypsin's substrates. Trypsin's narrow specificity is determined by the acyl moiety ($R-\overset{O}{\overset{\|}{C}}-$) and the type of bond cleaved is defined by the nature of X. Trypsin acts on peptide, amide or ester bonds (Walsh and Wilcox, 1970). The optimum pH for this reaction lies between pH 7 and 9 (Keil, 1971).

The following equation describes the reaction mechanism of trypsin (Keil, 1971): $E + S \overset{K_s}{\rightleftharpoons} ES \xrightarrow{k_2} ES' + P_1 \xrightarrow{k_3} E + P_2$. ES represents the enzyme-substrate complex, ES' the acyl-enzyme intermediate, $P_1$ the leaving group of the substrate and $P_2$ the carboxylic acid. The acylation step ($k_2$) is a nucleophilic reaction dependent on serine residue 183 and histidine residue 46 of trypsin (Bender and Kézdy, 1964). Serine residue 183 and histidine residue 46 apparently represent a charge relay system (Keil, 1971). Bender et al. (1964) reported that the acyl-enzyme intermediate is an ester of the substrate carboxyl and the hydroxyl of serine residue 183 of trypsin. Modification of histidine residue 46 leads to loss of the unusual reactivity of serine 183 (Breeley and Neurath, 1968). Some form of interaction exists between the hydroxyl group of serine residue 183 and the imidazole nitrogen of histidine residue 46 to account for the unusual reactivity of serine. The deacylation step ($k_3$) is also a nucleophilic reaction dependent on histidine residue 46 of trypsin.

The specificity site of trypsin presumably contains the negatively charged aspartic acid residue 177, that electrostatically binds positively charged groups of substrates and inhibitors (Smith and Shaw, 1969; Hartley, 1970).

The binding site of trypsin may be located between the specificity and catalytic site (Keil, 1971). Hydrophobic interactions between the carbon side chains or rings of substrates or inhibitors may occur at the binding site. Trypsin may possess two binding sites close to each other, one that binds charged molecules and one that binds neutral molecules (Sanborn and Bryan, 1968; Sanborn and

Hein, 1968).

Trypsin is most stable around pH 3 (Walsh and Wilcox, 1970). Trypsin can be stored for weeks at low temperatures without loss of activity. Four different forms of trypsin stored at low temperatures have been reported (Lazdunski and Delaage, 1967). Reversible transformations between these forms are pH dependent. Below pH 8, an increase in temperature leads to reversible denaturation. Above pH 8, irreversible denaturation is induced at elevated temperatures. Porcine trypsin is more stable than bovine trypsin to thermal destruction (Lazdunski and Delaage, 1965).

Autolysis of trypsin solutions can be retarded by the addition of calcium ions (Lazdunski and Delaage, 1965). A conformational change in the trypsin molecule resulting in a more compact structure is induced by calcium. The stabilizing effect of calcium ions is much greater with bovine and ovine trypsin than with porcine trypsin (Buck et al., 1962).

The activity of trypsin can be inhibited by proteinase inhibitors. The Kunitz and the Bowman-Birk inhibitors are the primary trypsin inhibitors isolated from soybeans (Kunitz, 1946; Bowman, 1946; Birk, 1961). The Kunitz inhibitor has been most thoroughly characterized. It has a molecular weight of about 21,500 (Wu and Scheraga, 1962). It is a globular protein consisting of a single polypeptide chain crosslinked by two disulfide bridges (Wu and Scheraga, 1962; Steiner, 1965). The isoelectric point of the Kunitz inhibitor is pH 4.5 (Kunitz, 1947).

The inhibition of trypsin by the Kunitz inhibitor is stoichiometric (Laskowski and Laskowski, 1954). The inhibition is competitive with the artificial substrate, benzoyl-L-arginine ethyl ester (Green, 1953). However, the type of inhibition is uncertain with protein substrates (Green, 1953). An arginyl-isoleucine bond is hydrolyzed when the inhibitor reacts with trypsin (Ozawa and Laskowski, 1966). This bond lies within one of the disulfide bridges of the Kunitz inhibitor. DiBella and Liener (1969) reported that only one of the disulfide bridges is essential for activity. At pH 4.0, partial resynthesis of the bond by trypsin was observed (Niekamp et al., 1969). An equilibrium mixture of 14% native and 86% cleaved form of trypsin inhibitor was reported.

The Bowman-Birk inhibitor associates reversibly in aqueous solutions to form a monomer, dimer and trimer mixture (Harry and Steiner, 1969; Millar et al., 1969). This explains why Birk et al. (1963) first reported that the molecular weight of the inhibitor was 20,000-24,000. Harry and Steiner (1969) and Millar et al. (1969) reported that the molecular weight of the Bowman-Birk inhibitor is approximately 8,000, which agrees with the minimum molecular weight estimated from the amino acid residues in the inhibitor (Frattali, 1969).

The inhibition of trypsin by the Bowman-Birk inhibitor has been reported to be noncompetitive and nonstoichiometric (Birk, 1968; Frattali, 1969). The lysine-X bond of the inhibitor was reported to be hydrolyzed by trypsin at pH 3.75 (Birk et al., 1967). The hydrolyzed inhibitor became less efficient

but retained its activity. Frattali and Steiner (1969) reported that the Bowman-Birk inhibitor was partly reconverted to its native form when incubated with equimolar amounts of trypsin.

The use of a pH stat to follow proteolysis

The theoretical principles involved in the construction and operation of a pH stat have been reviewed (Jacobsen et al., 1957). A pH stat consists principally of 1) a titration assembly, 2) a pH meter, 3) a titrator, 4) an autoburette and 5) a recorder. The titration assembly is made up of a temperature controlled reaction vessel with a magnetic stirrer, fitted with delivery tubes for the titrant and for the inert gas. The pH meter monitors the pH of the reaction mixture. The function of the titrator is to start the motor driven autoburette when the output potential of the pH meter deviates from a preset value and to stop it when the potential is restored to the original value. The autoburette adds either acid or base to the reaction mixture to achieve and maintain the pH at the preset value.

The titrator has a proportional band setting that determines the pH value at which the relay will operate the burette motor. Within an adjustable pH range (proportional band) near the set endpoint, the burette motor will run discontinuously with smaller and smaller current impulses, as the pH comes closer to the endpoint. The motor will completely stop when the endpoint is reached. Outside this range the motor will run continuously at maximum speed. Proportional control prevents "overshooting" of the endpoint. However, in practice the set endpoint will never

be reached. The working pH will deviate more from the set endpoint as the rate of the reaction studied increases. The difference between the working pH and the set endpoint can be reduced by choosing the smallest possible proportionality band of the instrument and by proper selection of the gear for the burette motor. Jacobsen et al. (1957) reported that deviations due to proportional control could be kept within 0.03-0.05 pH units if the maximum rate of adding reagent was at least two or three times the maximum rate required for the reaction.

The autoburette unit is based on the principle of displacement. A plunger is driven upward in an airtight chamber filled with titrant. As the plunger moves upward, titrant flows through a delivery tube into the reaction vessel. The autoburette has a speed selector that is used to change the motor speed and the rate of flow. The positions 1, 2, 4, 8 and 16 correspond to delivery of approximately 10, 20, 40, 80 and 160% of the total burette volume per minute. A counter registers the revolutions of the motor that drives the plunger. The uptake of reagent can be read directly from the counter that is calibrated in milliliters or recorded on a mechanical recorder. The recorder pen is driven synchronously with the plunger of the burette by a flexible shaft from the burette motor. The displacement of the pen on the ordinate is proportional to the amount of reagent delivered by the autoburette. A synchronous motor moves the strip chart to provide a time scale on the abscissa.

The rate and extent of proteolysis can be determined by measuring the production of hydrogen ions. Proteolysis in aqueous solutions can be followed

with some degree of accuracy by means of a pH stat if the working pH is kept within certain limits (Jacobsen et al., 1957). In acid solutions, the pH should not exceed the pK of the carboxyl group by more than one pH unit, and it should not be lower than pH 3. In alkaline solutions, the pH should not be more than one pH unit below the pK value of the amino group and should not be higher than pH 11. The pK values of the carboxyl and amino groups are about 2-4 and 7-10, respectively. The acid range is much narrower than the alkaline range.

Waley and Watson (1953) were the first to follow the hydrolysis of the peptide bond by continuous titration at a constant pH. They studied tryptic hydrolysis of polylysine at pH 7.6. The amount of alkali taken up was proportional to the number of peptide bonds split (Waley and Watson, 1953). The reaction was zero order throughout a considerable part of the hydrolysis.

Samuelson and Li (1964) calculated the number of peptide bonds hydrolyzed by trypsin in ovine prolactin from the alkali uptake in the pH stat and the pK of the $\alpha$-amino groups liberated. Good agreement was reported between the number of bonds calculated from the pH stat data and the number of $\alpha$-amino groups estimated by dinitrofluorobenzene. Mihalyi and Harrington (1959) also reported good agreement between the pH stat data and the nitrofluorobenzene technique for trypsin hydrolysis of myosin. In another study, von Hippel et al. (1960) found that digestion of collagen followed both by the pH stat and by estimation of $\alpha$-amino groups by a colorimetric ninhydrin

method produced similar results. The pH stat has been reported to be sensitive enough to follow the hydrolysis of a single peptide bond in the ovalbumin molecule during its conversion into plakalbumin by subtilisin (Ottesen, 1956).

Lynch et al. (1977) used the pH stat to monitor alkali uptake during tryptic hydrolysis of glycinin and its subunits at pH 8.0, 25°C. Native glycinin, acid denatured (pH 2) glycinin, alkali denatured (pH 12) glycinin, carboxyamidemethyl (CAM) glycinin, CAM acidic subunits and CAM basic subunits were examined. The reactions were allowed to proceed until no further uptake of base was observed. The slopes of the curves of relative hydroxyl ion uptake with time were used to compare reaction rates. Alkali denatured glycinin and the CAM acidic subunits exhibited the fastest rates of hydrolysis, followed very closely by CAM glycinin. Intermediate rates were shown by the native and acid denatured glycinin. The CAM basic subunits were hydrolyzed at the slowest rate.

Some sources of error associated with the pH stat have been reviewed (Jacobsen et al., 1957; Mihalyi, 1978). The base line of the reaction before enzyme addition must be established when estimating the base uptake during enzymatic hydrolysis. Drifts in the base line can be caused by instability of the instrumental electronics, electrodes or chemical system used (Mihalyi, 1978). Electronic stability is not usually a problem. However, proper grounding and good temperature control are necessary for a drift free system. Combined

electrodes may cause a stability problem. A change in the liquid junction potential between the saturated KCl in the reference electrode and the reaction mixture is usually the problem. High salt concentrations may lead to protein precipitating on the reference electrode, blocking the fiber and porous plug junction. Mihalyi (1978) suggested using a ground glass, sleeve-type electrode for this problem. Samples are usually made up in 0.1 M KCl to insure an effectively constant ionic strength (Waley and Watson, 1953; Jacobsen et al., 1957).

Proteins have also been reported to coat the glass electrodes, thus causing drifts in potentials (Jacobsen et al., 1957; Kenchington, 1960; Mihalyi, 1978). Kenchington (1960) reported that storage of the glass electrode in 0.1 M HCl for up to 48 hours was effective in removing the protein.

Ballantyne (1968) emphasized the importance of an inert atmosphere in the reaction vessel when using alkali titrant during pH stat recording. He reported that acidic substances from the air resulted in a slow continuous addition of alkali to the reaction vessel. The rate of alkali uptake increased with a decrease in hydrogen ion concentration. For example, 1.25 μmoles NaOH/hr were added at pH 7.5, 1.70 at pH 8 and 2.05 at pH 8.5. When $CO_2$ was introduced into the vessel, continuous addition of alkali occurred due to $H_2CO_3$ formation. When pure $N_2$ was introduced, the addition of titrant ceased. Ballantyne (1968) suggested passing the nitrogen through $CO_2$ free distilled water to prevent evaporation of the reaction mixture, especially during prolonged titrations.

Traces of ammonium sulfate, frequently used in preparing enzymes and substrates, may lead to instability of the base line (Mihalyi, 1978). At an alkaline pH, $NH_3$ is removed from the $N_2$ gas stream, leading to base line drifts which are proportional to the gas flow rate.

If all the previous precautions fail in obtaining a stable base line, Mihalyi (1978) suggested estimating base line drift by recording alkali uptake for a period of time prior to the addition of the enzyme. He predicted reproducibility to within 1%.

Determining the point of origin of the rate curve is another source of error in evaluating pH stat data (Mihalyi, 1978). Often the pH of the enzyme is not adjusted to the reaction pH to avoid its inactivation by self-digestion. Although a small amount of acid is introduced with the enzyme, this may be negligible if the substrate concentration is high. However, it may have a significant effect if the substrate concentration is low or if an initial fast reaction occurs involving only a few peptide bonds. A separate blank experiment can be used to evaluate these effects.

Adequate stirring is important in obtaining smooth rate curves (Jacobsen et al., 1957; Mihalyi, 1978). A dye can be used to determine if there is enough vertical and circular mixing.

Jacobsen et al. (1957) pointed out that the tip of the delivery tube from the autoburette should be as narrow as possible to prevent unregistered titrant from leaking into the reaction mixture due to convection currents, especially if the

titrant has a slightly higher density than the reaction mixture. When the motor starts adding titrant again, the titrant would be contaminated with the reaction mixture at first, resulting in a discontinuous rate curve. Two possible solutions to this problem are using a titrant with a lower density than that of the reaction mixture and using a delivery tube tip which has a small U-bend (Jacobsen et al., 1957).

# MATERIALS AND METHODS

## Soy Flour

Three samples of soy flour with different heat treatments were obtained from Archer Daniels Midland Company, Decatur, Illinois. Toasted Nutrisoy Flour (sample A), Toasted Nutrisoy Flour 40 (sample B) and Nutrisoy 7B Flour (sample C) were stored at 4° C in polyethylene self-sealing bags. Sample A had received the most heat treatment, while sample C had received the least heat treatment.

## Chemical Analyses

### Moisture content

Triplicate 2 g samples of soy flour were analyzed for moisture content using the vacuum oven method (14.003, A.O.A.C., 1975). The samples were placed in tared, aluminum weighing dishes, weighed and dried at 98° C at a vacuum of 25 inches of mercury for 5 hours. The samples were transferred to a desiccator, cooled and weighed. Moisture content was calculated as weight loss.

### Crude lipid content

Crude lipid was determined by petroleum ether (Skelly B) extraction using a Goldfisch extraction apparatus according to the procedure of A.A.C.C. (1969) method 30-20. Duplicate, 5.0 g, dry samples were placed into dry, tared

extraction thimbles. The samples were weighed and extracted for 16-18 hours. Excess solvent was evaporated from the thimbles under a hood. The samples were dried in an oven at 100°C for 30 minutes to remove any remaining traces of solvent, placed in a desiccator, cooled and weighed. The crude lipid was calculated as weight loss due to extraction.

Protein content

The nitrogen content was determined by a modification of the micro-Kjeldahl procedure 47.021 (A.O.A.C., 1975). Protein content was calculated using the conversion factor 6.25.

Triplicate 0.1 g samples were digested with 2 ml of concentrated sulfuric acid on a Lab Con Co. digestion apparatus. Cupric selenite (0.2 g) was used as a catalyst for the reaction, and $K_2SO_4$ (0.3 g) elevated the boiling point. The samples were digested for approximately 30-45 minutes, then the necks of the digestion flasks were rinsed with a small amount of acid. Digestion was continued for an additional 30 minutes.

After the samples were digested, they were allowed to cool to room temperature. Distilled water (5 ml) was added to dissolve the solid material. The mixture was quantitatively transferred to a Lab Con Co. distillation apparatus. Excess NaOH solution, 40% (w/v), was added to release the ammonia. The ammonia was collected in 10 ml of 4% boric acid and measured by titration with 0.1 N HCl to a gray endpoint using Tashiro's indicator. Tashiro's indicator was prepared by

dissolving 0.25 g methylene blue and 0.375 g methyl red in 300 ml of 95% ethyl alcohol.

Protein dispersibility index

Protein dispersibility index (PDI) was determined by a modification of the procedure of A. A.C.C. (1969) method 46-24. Triplicate 20 ± 0.1 g samples were quantitatively transferred to blender cups containing 50 ml of distilled water. The flour and water were stirred to form a paste with a spatula. Distilled water (250 ml) was added in increments to form a smooth slurry. The last increment of water was used to rinse the spatula and blender cup walls. After 10 minutes of blending at high speed, the slurry was poured into a 600 ml beaker. The slurry was allowed to separate at room temperature for 30 minutes. A 25 ml portion of the top layer was centrifuged at 930 x g for 10 minutes. Water dispersible nitrogen in the supematant was determined by the micro-Kjeldahl procedure previously described. PDI was calculated as follows:

$$\text{PDI} = \frac{\text{\% water dispersible protein} \times 100}{\text{\% total protein}}$$

Trypsin inhibitor assay

Trypsin inhibitor activity of the soy flours was determined by the method described by Kakade et al. (1974). One gram samples of soy flour were extracted with 50 ml of 0.01 N NaOH for 1 hour to remove the trypsin inhibitors. The suspensions were diluted to 0.3 mg soy flour/ml. Duplicate portions (0, 0.6,

1.0, 1.4 and 1.8 ml) of the diluted soy flour suspensions were pipetted into test tubes and made up to 2.0 ml with distilled water. A trypsin solution was prepared by dissolving 4 mg of trypsin (Type IX, Sigma Chemical Company) in 200 ml of 0.001 N HCl. Two milliliters of trypsin solution were added to each test tube. Benzoyl-DL-arginine-p-nitroanilide (BAPA, Sigma Chemical Company) was the substrate. BAPA was prepared by dissolving 40 mg in 1 ml of dimethyl sulfoxide and diluting to 100 ml with tris buffer (0.05 M, pH 8.2). The reaction was started by adding 5 ml of the substrate to the reaction mixture and was stopped after 10 minutes at 37° C, by the addition of 1 ml of 30% acetic acid. The contents of each tube were filtered through Whatman No. 3 filter paper, and p-nitroaniline was measured by absorbance at 410 nm against a reagent blank. The reagent blank was prepared by adding 2 ml of water, 2 ml of trypsin solution, 1 ml of 30% acetic acid and 5 ml of BAPA solution to a test tube in the order listed. Tryptic hydrolysis of BAPA follows a zero order reaction rate, and a linear relationship exists between the quantity of p-nitroaniline released and the concentration of trypsin.

Trypsin inhibitor activity was expressed as trypsin units inhibited per milligram of soy flour (TUI/mg). One trypsin unit (TU) was arbitrarily defined as an increase in absorbance of 0.01 at 410 nm per 10 ml of the reaction mixture under the described conditions.

### Tryptic Hydrolysis of Soy Proteins

A modification of the in vitro digestibility method described by Hsu et al. (1977) was followed. A pH stat was used to determine the initial reaction rates

of tryptic hydrolysis of soy flour. The pH stat measures protein hydrolysis as a function of time by automatically titrating the hydrogen ions produced as the reaction proceeds at a constant pH. Since enzyme activity is markedly affected by pH, this modification should be an improvement over the method described by Hsu et al. (1977), that involves correlating the pH decrease after 10 minutes of hydrolysis to digestibility.

## Preparatory procedures

Substrate dispersions Varying quantities of soy flour (2.4, 2.8, 3.0, or 3.2 g, nearest 0.1 mg) were added to 125 ml of 0.2 M KCl. The soy flour mixtures were stirred at room temperature for 30 minutes, adjusted to pH 9.0, then stirred an additional 30 minutes. The pH was readjusted to pH 9.0, and the samples were diluted to 250 ml with deionized distilled water. The final soy flour concentrations were 0.96, 1.12, 1.20 or 1.28 g/100 ml of 0.1 M KCl. These concentrations correspond to 0.50, 0.58, 0.62 and 0.67 g soy protein/100 ml of 0.1 M KCl, respectively.

BAEE solutions A solution of 75.0 mg $\alpha$-N-benzoyl-L-arginine ethyl ester (BAEE, Sigma Chemical Company) was prepared in 125 ml of 0.2 M KCl. The solution was diluted to 250 ml with deionized distilled water and stored at 4° C for up to 2 weeks. Tryptic hydrolysis of BAEE was determined at the beginning and end of each

experiment to ensure the reproducibility of the procedure. Trypsin hydrolyses the ester bond of BAEE forming benzoylarginine and ethanol. One mole of hydrogen ion is released for each mole of BAEE hydrolyzed. The rate of acid production is constant throughout more than 90% of the hydrolysis (Walsh and Wilcox, 1970).

Trypsin solutions Porcine trypsin (Type IX, Sigma Chemical Company, 14,350 BAEE units/mg protein) was dissolved in deionized distilled water and maintained in an ice bath during use. It retained its initial activity when stored at 4°C for at least 2 weeks.

Trypsin inhibitor In the experiments determining the effect of trypsin inhibitor on the reaction rate, the inhibitor was added to the soy flour suspensions prior to the initial mixing (Sigma Chemical Company; 1 mg of inhibitor will inhibit 1.5 mg of trypsin with 10,000 BAEE units/mg protein).

Standard NaOH solutions The solutions were standardized with potassium acid phthalate and stored with soda lime protection to prevent changes due to $CO_2$ uptake. Three concentrations of NaOH were used: 0.001 N, 0.0147 N and 0.0248 N.

pH stat

Apparatus A London Radiometer pH stat was used. It consisted of a titrigraph, type SBR2c; titrator, type TTT 11; 0.25 ml autoburette, type ABUlc;

titration assembly, type TTA31; pH meter, type PHM 26; glass electrode, type G2222B; and Calomel electrode, type K4112. The titrations were done in thermostatted vessels equipped with magnetic stirrers. A stream of $N_2$ excluded $CO_2$ from the reaction vessel. The $N_2$ was passed through $CO_2$ free distilled water in a wash bottle to prevent evaporation of the vessel contents.

Instrumental parameters The following instrumental parameters were used: temperature, 25° C; endpoint, pH 9.0; proportional band setting, 0.05, 0.1 or 0.2; speed, 8 or 16; chart gear A, 10 mm/rev; chart motor B, 4 rev/min; and CXD (% of syringe capacity/min), 1%.

Procedure for typical kinetic run Soy flour substrate (5 ml) was pipetted into the reaction vessel. After a small amount of water was placed between the vessel and the jacket to aid in heat transfer, the vessel was placed in position in the titration assembly. The speed of the magnetic stirrer was adjusted so that the stirring was sufficient to cause rapid flow of the solution past the electrodes. Constant temperature water (25° C) was circulated through the jacket of the reaction vessel. Small decreases in the pH of the stock substrate solution occurred with time, probably due to $CO_2$ uptake. The substrate solution was readjusted to pH 9.0 in the pH stat by adding a small amount of titrant. The pH stat was run for 5 minutes while the temperature and the pH of the sample equilibrated. Small drifts occurred in the system, even though a stream of $N_2$ excluded $CO_2$ from the reaction vessel. These small decreases in pH were not a problem, unless a proportional band setting of 0.05

was used. At this setting the system became too sensitive, resulting in discontinuous rate curves that were not reproducible.

A microsyringe was used to add the enzyme solution. Care was taken to ensure that all of the bubbles were out of the syringe. The enzyme was introduced beneath the surface of the solution in the titration vessel. The reaction was allowed to proceed for approximately 2-3 minutes. When the run was completed, the autoburette was refilled, the reaction mixture was removed and discarded, and the electrodes and stirrer were rinsed. The reaction vessel was cleaned and dried between each run.

The glass electrode was soaked 24 hours in 0.1 M HCl and 24 hours in pH 7.0 buffer after each days use. Use of two or more electrodes made it possible to use the pH stat daily. Soaking the electrodes was found to be necessary to maintain the stability of the pH meter, especially when high substrate concentrations were used.

The tip of the delivery tube was taken out of the reaction vessel and covered overnight. Otherwise the first few samples showed unusually high rates due to buffer mixing with titrant in the delivery tube.

Interpretation of recorder tracings The "initial" rates were determined from the slopes of the essentially linear portions of the titration plots in the initial one minute period. Time and volume data could be taken directly from the charts. The volume information could be transformed directly into nmol by multiplying by

the base concentration. "Initial" rates were calculated in terms of nmol NaOH/sec.

Under optimum conditions the linear portion of the titration plot should cover at least 50% of the chart paper, to ensure accurate determination of the slope. By adjusting the burette size, NaOH concentration and chart speed, these optimum conditions could be approached.

# RESULTS AND DISCUSSION

## Factors Affecting the pH Stat Method

### Temperature

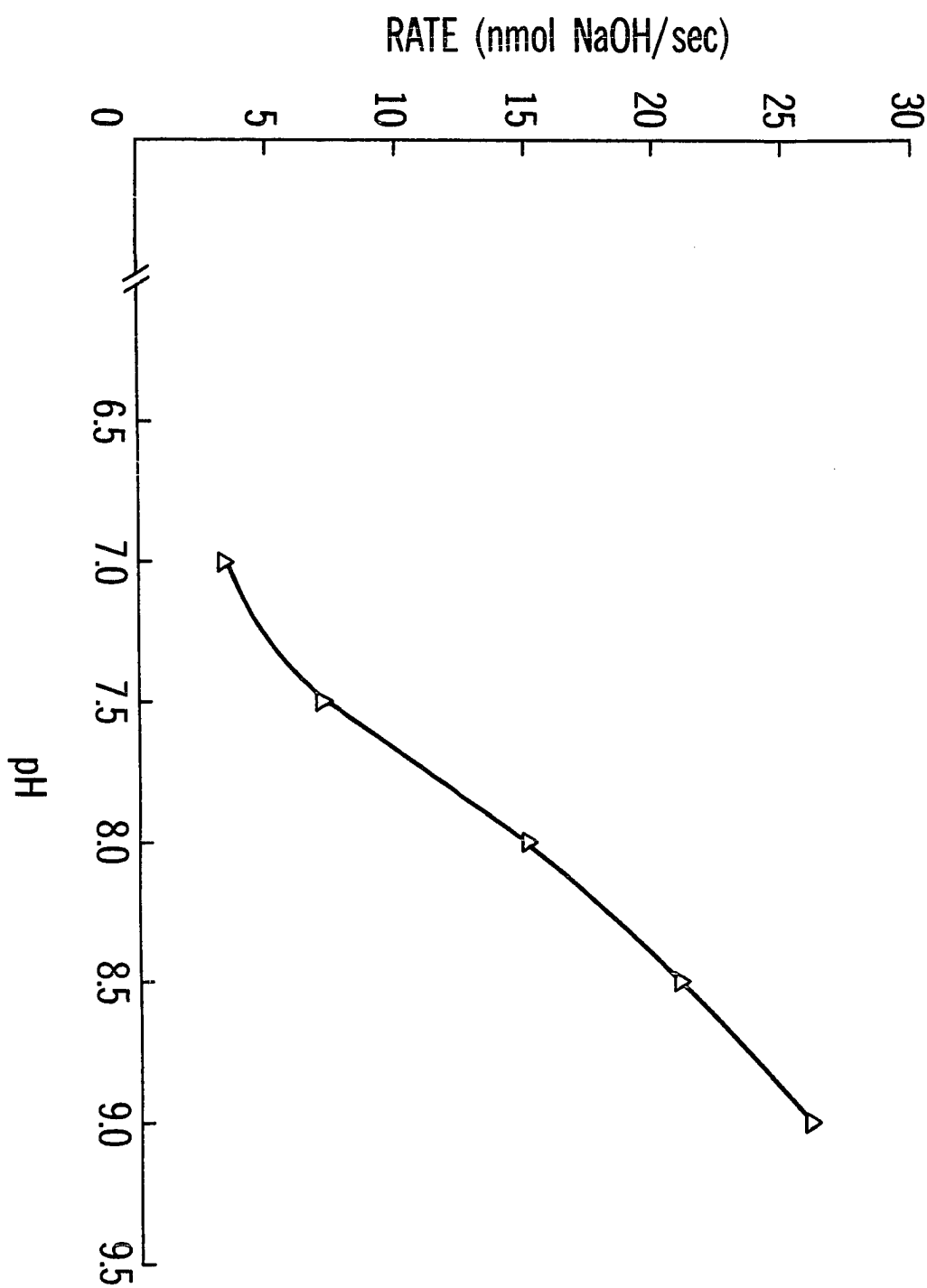
In this study, tryptic hydrolysis of soy protein was followed by a pH stat at 25° C. Although a temperature controlled reaction vessel was used, it was convenient to keep the titrant at room temperature (approximately 25° C). Some initial experiments were done at 37° C; however, proteolysis was measured at 25° C in most of the experiments to ensure a minimum temperature deviation due to added titrant.

### pH

The effect of pH on the initial reaction rate of tryptic hydrolysis of soy flour, determined by the pH stat method, is shown in Figure 1. The pH stat monitors tryptic hydrolysis of protein by titrating the hydrogen ions that are released from the newly formed amino groups. The number of hydrogen ions produced is proportional to the number of peptide bonds split. The following equilibrium exists: $— NH_3^+ \rightleftharpoons — NH_2 + H^+$. As the pH increases from one unit below to one unit above the pK, in increments of one, the percentage of hydrogen ions present in the equilibrium changes from 9 to 50 to 90.9 (Mihalyi, 1978). The pK of an amino group in an amino acid is around 9, but it is lowered to about 8 when the group exist as an N-terminal group (Tanford, 1962). Assuming that

Figure 1. Effect of pH on initial rates of tryptic hydrolysis of sample A (1.19 g/100 ml)

The experimental parameters were: 37° C, 0.001 N NaOH, proportional band 0.1, speed 8, 0.8 mg bovine trypsin


RATE (nmol NaOH/sec)
0
5
10
15
20
25
30
pH
6.5
7.0
7.5
8.0
8.5
9.0
9.5

the average pK value of the newly formed amino group is about 8, the reaction rates at pH 7 and 9 should be about 2.7 and 27 nmol NaOH/sec, respectively, due to a shift in the hydrogen ion equilibrium. The rates shown in Figure 1 indicate that the change with pH is due mainly to dissociation. Deviations in the data from the theoretical values under these assumptions may be due to changes in trypsin activity with pH, and to buffering by existing amino groups.

Some initial experiments were done at pH 8; however, in all other experiments, proteolysis was measured at pH 9 because of increased rates and sensitivity.

At low substrate concentrations (0.1 g/100 ml), the pH of the enzyme solution had a significant effect on the reaction rate. The pH of the trypsin solution was not adjusted to pH 9 but maintained at pH 5.5 to prevent autolysis. The addition of the enzyme caused an immediate pH decrease at low substrate concentrations, resulting in an unusually fast initial reaction rate. At higher substrate concentrations (1 g/100 ml), the pH of the enzyme had very little if any effect on the reaction rate, since the buffering capacity of the system was much greater. The addition of $CaCl_2$ (stabilizing agent) to the trypsin solution also resulted in unusually fast rates at low substrate concentrations. The fast rates may be due to the formation of $Ca(OH)_2$, which would cause a pH decrease.

Substrate concentration

In a theoretical plot of initial rate of reaction versus substrate concentration, the rate increases with substrate concentration up to a maximum (Barrow, 1974). When the enzyme is completely saturated with substrate, no further increase in the rate occurs. At this substrate concentration, the rate is zero order and independent of substrate concentration.

A typical reaction curve obtained for the tryptic hydrolysis of soy flour, using a pH stat to monitor the reaction, is shown in Figure 2. During the first minute of the reaction, a linear relationship exists between the reaction rate and time. The initial rate appears to be zero order. The reaction rate may deviate from zero order with time as the substrate is used up, and trypsin is no longer saturated.

Figure 3 shows the effect of substrate concentration (soy flour) on the initial reaction rate at different enzyme concentrations. The initial reaction rates were calculated from the slopes of the linear portions of the reaction curves. The slopes of the curves in Figure 3, as well as all subsequent slopes, were calculated using linear regression. As the substrate concentration increased, the initial reaction rate decreased. For example, when 0.5 mg of trypsin was added to 0.96, 1.12 or 1.28 g soy flour/100 ml, the rates were 67, 42 or 27 nmol NaOH/sec, respectively. However, at any one substrate concentration, the rates were linear with enzyme concentration.

Figure 2. Typical reaction curve for tryptic hydrolysis of sample A (1.28 g/100 ml)

The experimental parameters were: 25°C, pH 9, 0.0147 N NaOH, proportional band 0.1, speed 16, 0.64 mg porcine trypsin

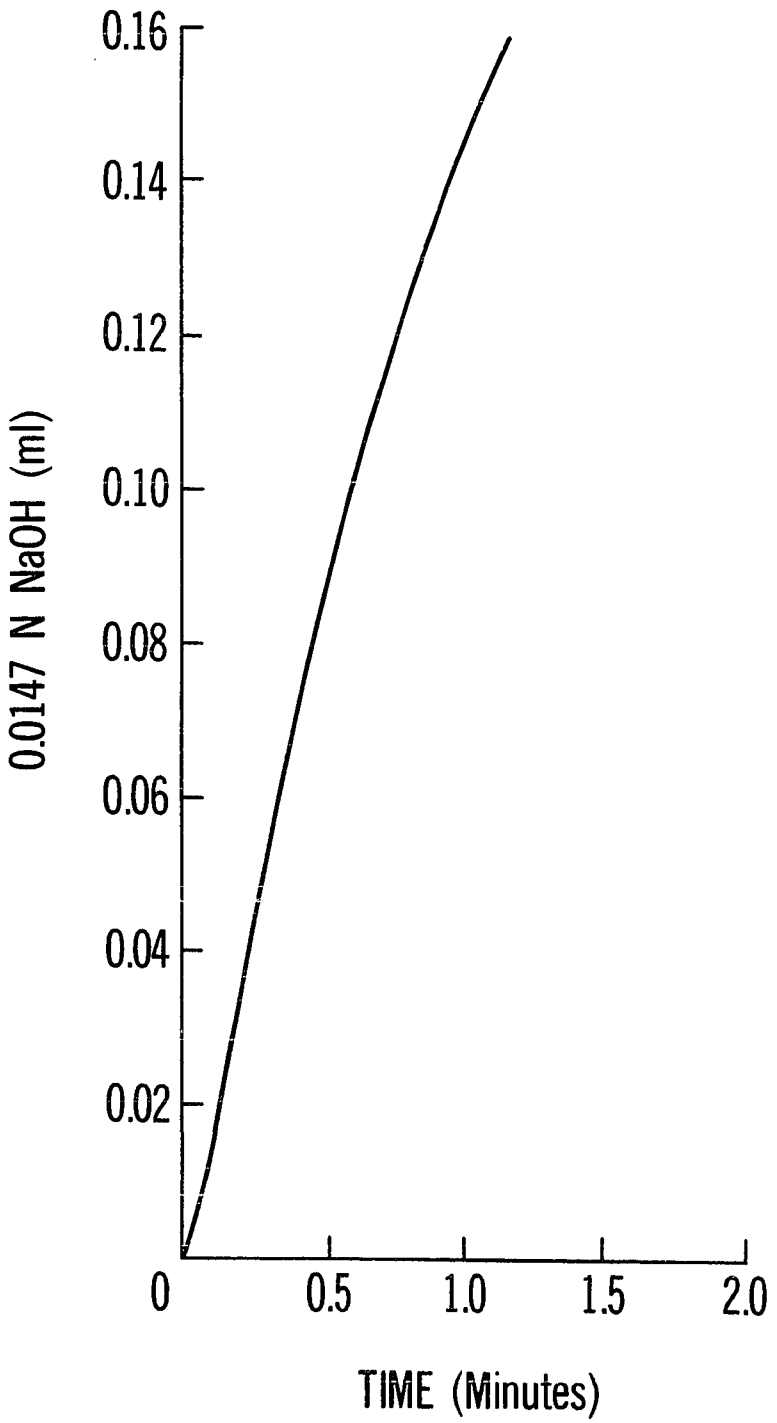
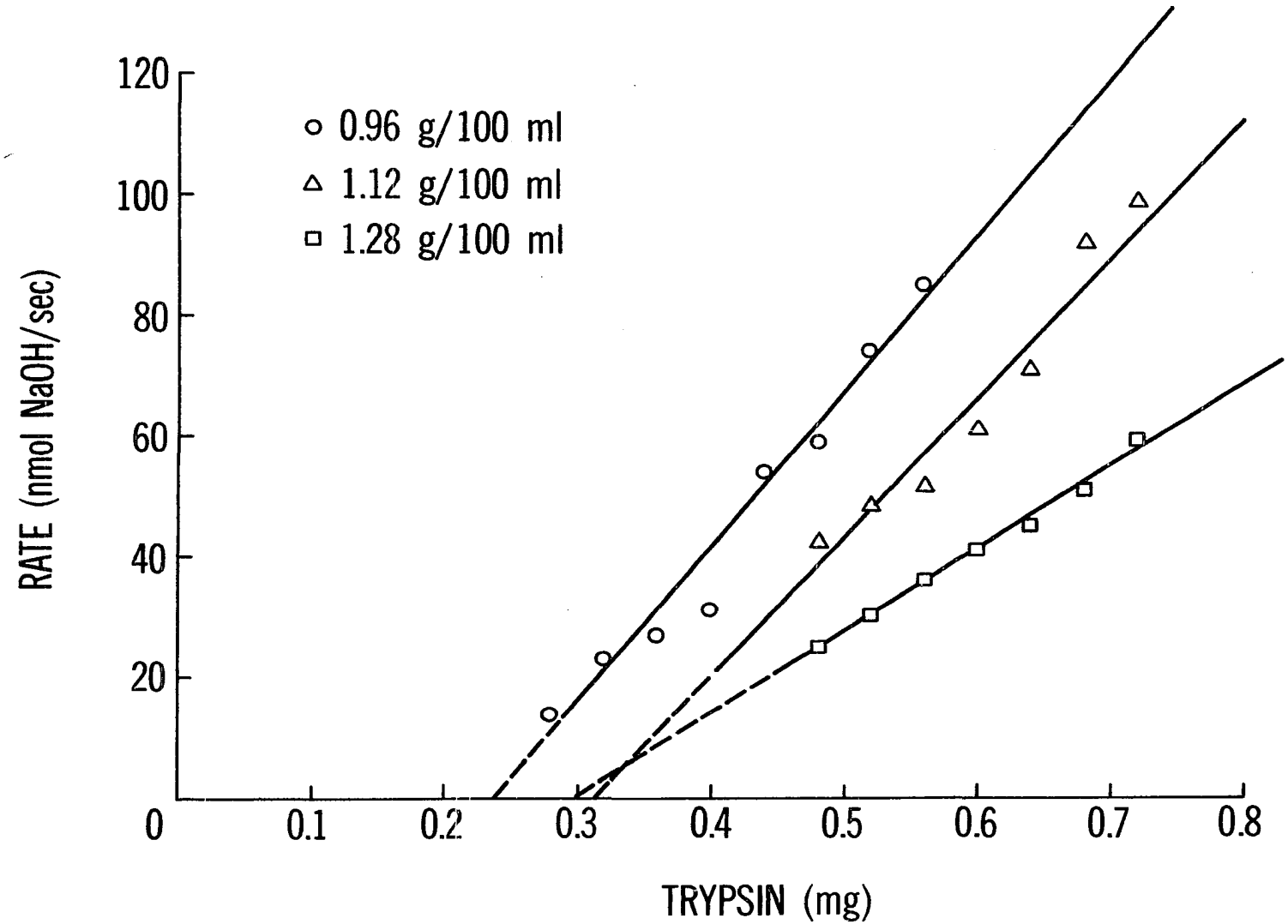

0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.0147 N NaOH (ml)
0
0.5
1.0
1.5
2.0
TIME (Minutes)

Figure 3. Effect of substrate concentration on initial rates of tryptic hydrolysis of sample A

The experimental parameters were: 25° C, pH 9, 0.0147 N NaOH, proportional band 0.1, speed 16


120
100
80
60
40
20
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
RATE (nmol NaOH/sec)
TRYPSIN (mg)
o 0.96 g/100 ml
Δ 1.12 g/100 ml
□ 1.28 g/100 ml

An explanation of this phenomena may be twofold. The trypsin inhibitor concentration as well as the buffering capacity of the system increases with substrate concentration. Since the pH stat measures the free hydrogen ions released during proteolysis, an increase in the buffering capacity would decrease the reaction rate. Preliminary experiments were run at a lower substrate concentration (0.1 g/100 ml) to try to minimize the effects of buffering. However, the reproducibility of the system decreased. The "noise" in the system could not be effectively separated from the reaction curve. Calculations of initial reaction rates from the discontinuous rate curves were very difficult.

In order to try to differentiate the effects of trypsin inhibitors and buffering on the reaction rates, tryptic hydrolysis of bovine serum albumin (BSA, Sigma Chemical Company) was followed. The initial rate of BSA hydrolysis increased with substrate concentration up to a maximum, where it levelled off and began to decrease (Figure 4). Both BSA and soy protein contain buffering groups. However, buffering seemed to play a role in the rate of proteolysis of BSA only at high substrate concentrations (2.0 g/100 ml). A combination of trypsin inhibitor activity and buffering capacity can explain the unusual relationship between substrate concentration and the rate of proteolysis of soy flour.

Although there is a linear relationship between enzyme concentration and the initial rate of hydrolysis of soy flour, the intercept of the line does not go through zero, as it does for BSA (Figures 3,5). The shift in the intercept may be due to trypsin inhibitor activity. Trypsin inhibitors can bind trypsin and

Figure 4. Effect of substrate concentration on initial rates of tryptic hydrolysis of BSA

The experimental parameters were: 25°C, pH 8, 0.001 N NaOH, proportional band 0.1, speed 8, 0.4 mg porcine trypsin

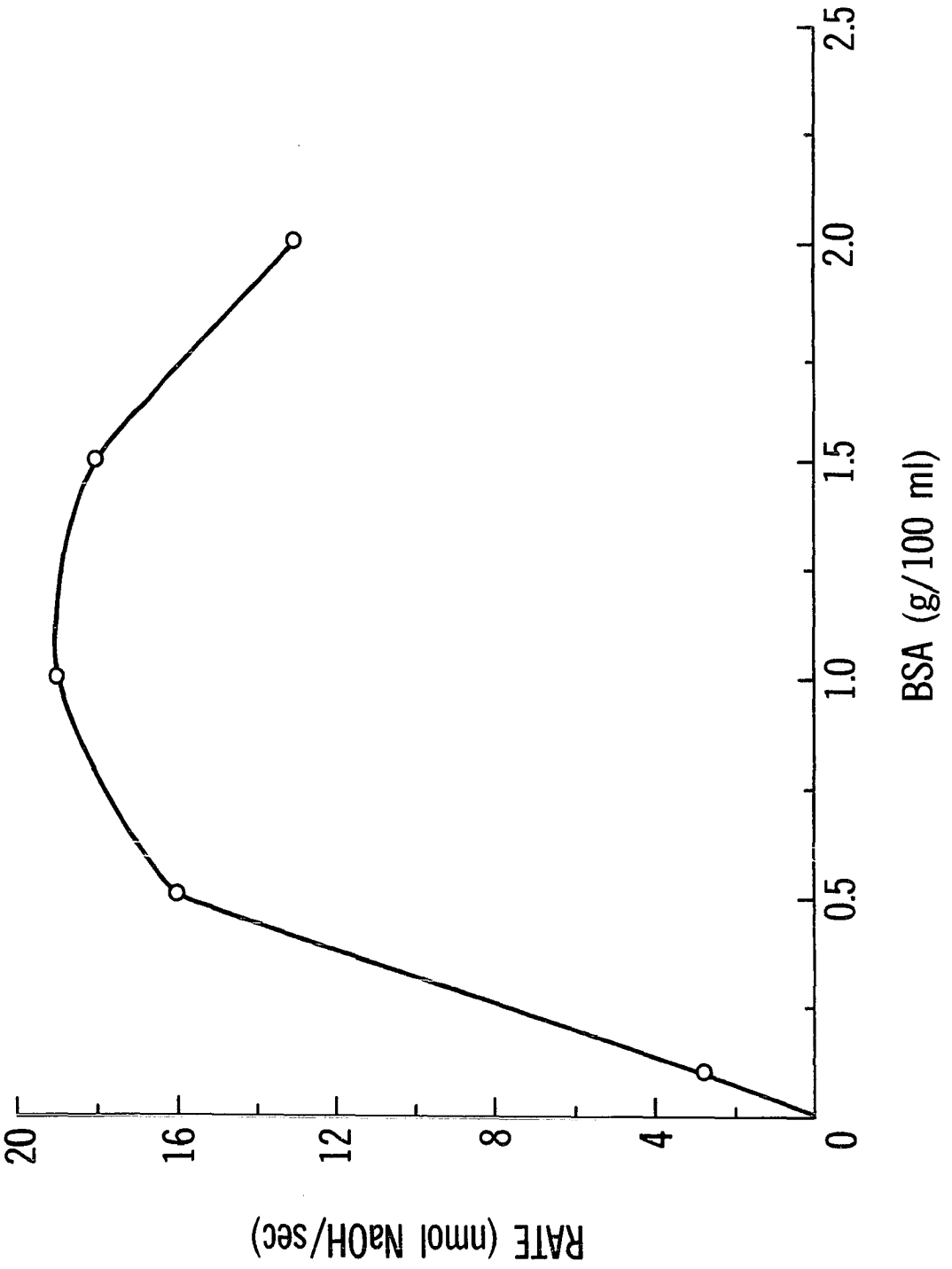
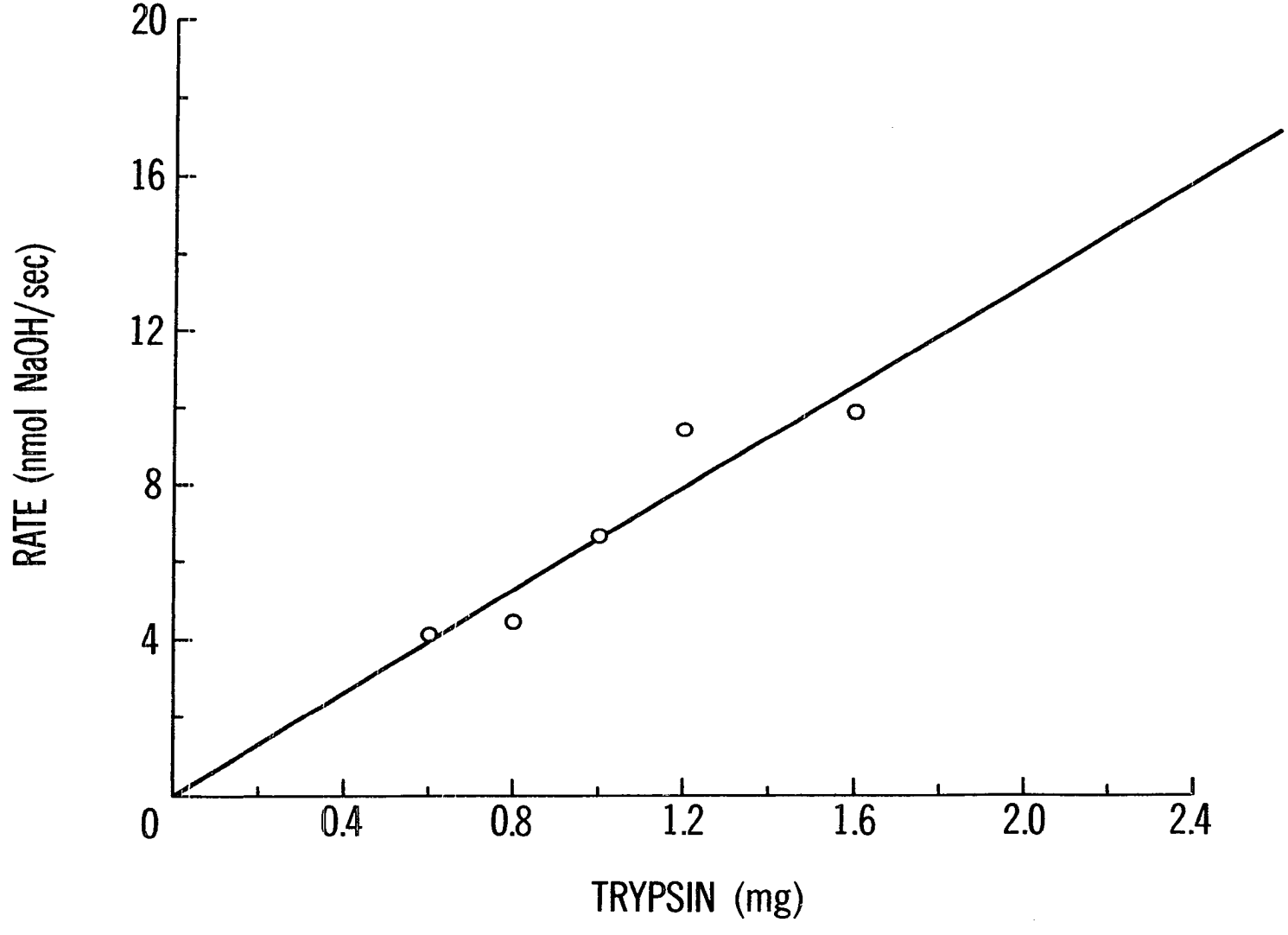

RATE (nmol NaOH/sec)
20
16
12
8
4
0
0.5
1.0
1.5
2.0
2.5
BSA (g/100 ml)

Figure 5. Effect of enzyme concentration on initial rates of tryptic hydrolysis of BSA (0.1 g/100 ml)

The experimental parameters were: 25° C, pH 8, 0.001 N NaOH, proportional band 0.05, speed 8


20
16
12
8
4
0
RATE (nmol NaOH/sec)
0.4
0.8
1.2
1.6
2.0
2.4
TRYPSIN (mg)

decrease or eliminate its activity.

In order to ensure that the experimental conditions were stable, tryptic hydrolysis of BAEE was determined at the beginning and end of each experiment. The reaction curves obtained using BAEE as the substrate were linear, since 90% of the reaction is zero order (Walsh and Wilcox, 1970). A typical reaction curve for BAEE is shown in Figure 6. The steps in the curve represent small overshoots of the endpoint. The size of the steps in the curve were influenced by the NaOH concentration, the speed setting and the proportional band setting. However, these factors did not influence the initial rate of BAEE hydrolysis. Trypsin hydrolyzes the ester bond of BAEE forming benzoylarginine and ethanol. One mole of hydrogen ion is released for each mole of BAEE hydrolyzed. For example, 0.14 nmol of NaOH was required to maintain a constant pH, when 5 ml of BAEE (0.1 mg/ml) containing 0.145 nmol was hydrolyzed to completion. Changes in BAEE concentration above the enzyme saturation level (0.002-0.03 g/100 ml) did not affect the reaction rate, since there are no groups present in BAEE to buffer the system. Also, benzoylarginine is not a strong inhibitor of the reaction (Walsh and Wilcox, 1970). A plot of the initial rate of proteolysis of BAEE versus trypsin concentration is shown in Figure 7.

## Instrumental parameters

### Proportional band

The effects of the proportional band setting on the initial reaction rates of soy flour samples A and B are shown in Figures 8 and 9.

Figure 6. Typical reaction curve for tryptic hydrolysis of BAEE (0.03 g/100 ml)

The experimental parameters were: 25° C, pH 9, 0.0248 N NaOH, proportional band 0.1, speed 16, 0.072 mg porcine trypsin

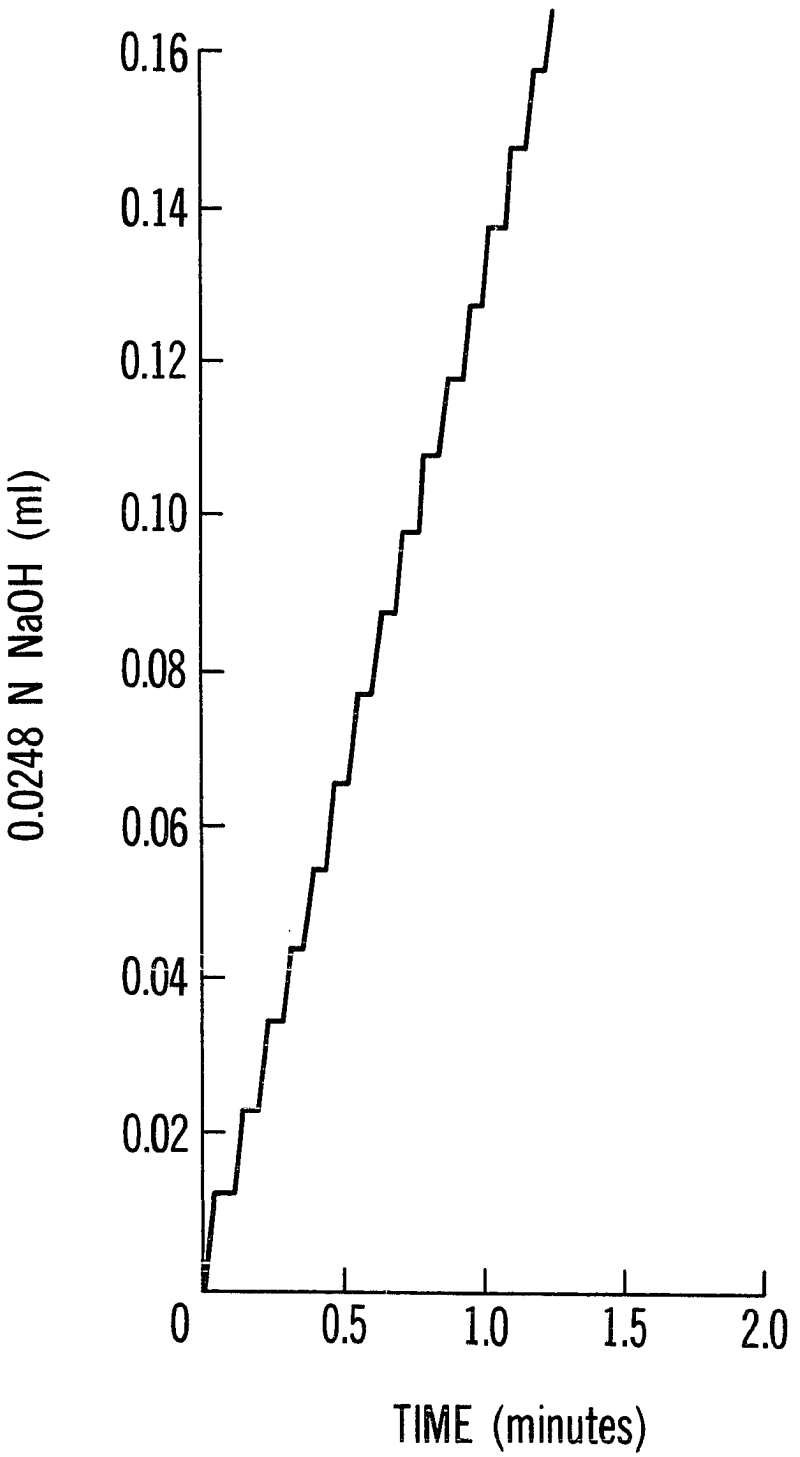

0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.0248 N NaOH (ml)
0
0.5
1.0
1.5
2.0
TIME (minutes)

Figure 7. Effect of enzyme concentration on initial rates of tryptic hydrolysis of BAEE (0.03 g/100 ml)

The experimental parameters were: 25°C, pH 9; NaOH concentration, proportional band and speed did not influence the rates

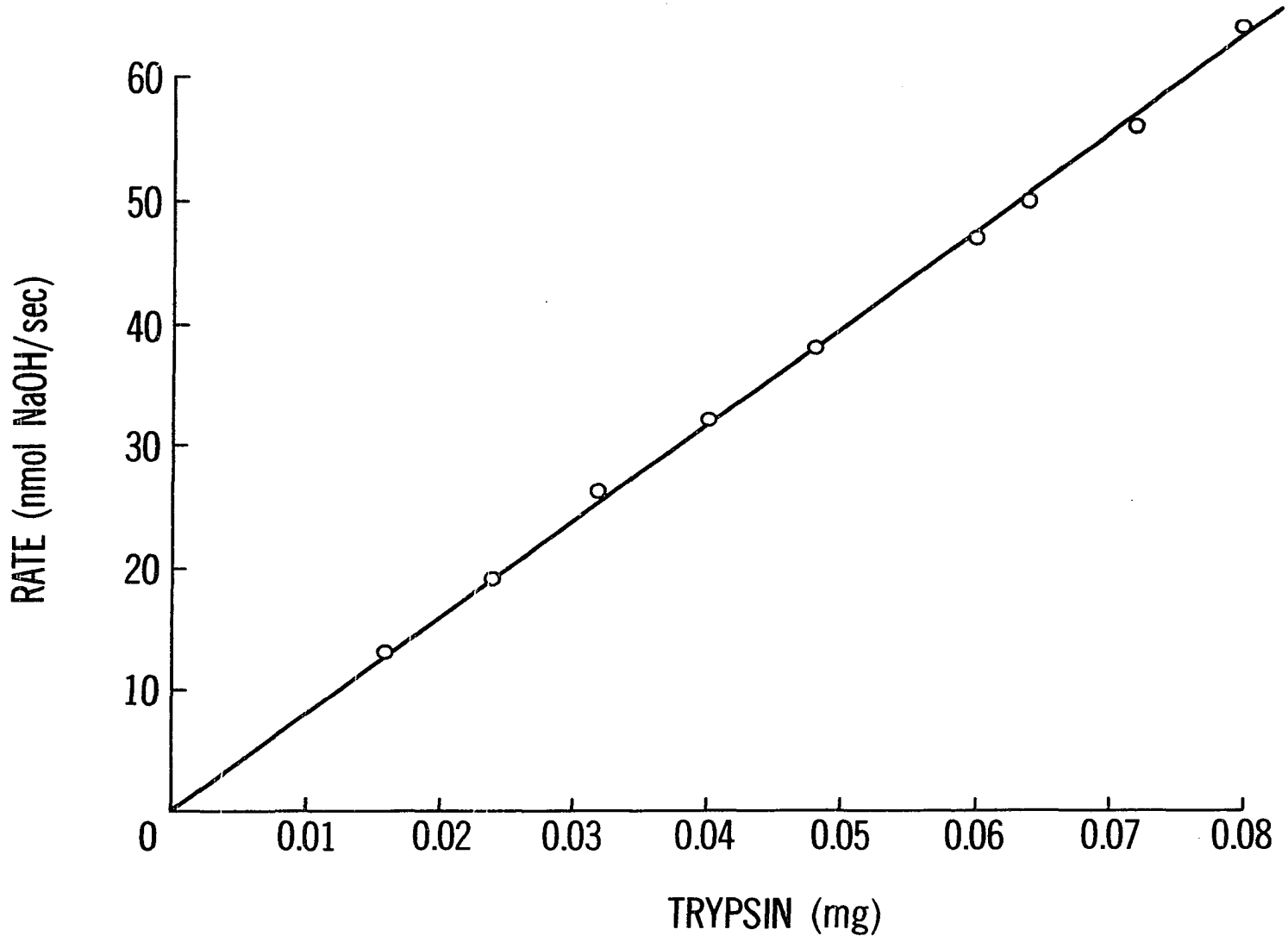

RATE (nmol NaOH/sec)
60
50
40
30
20
10
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
TRYPSIN (mg)

Figure 8. Effect of the proportional band setting on initial rates of tryptic hydrolysis of sample A (1.2 g/100 ml)

The experimental parameters were: 25° C, pH 9, 0.0147 N NaOH, speed 8

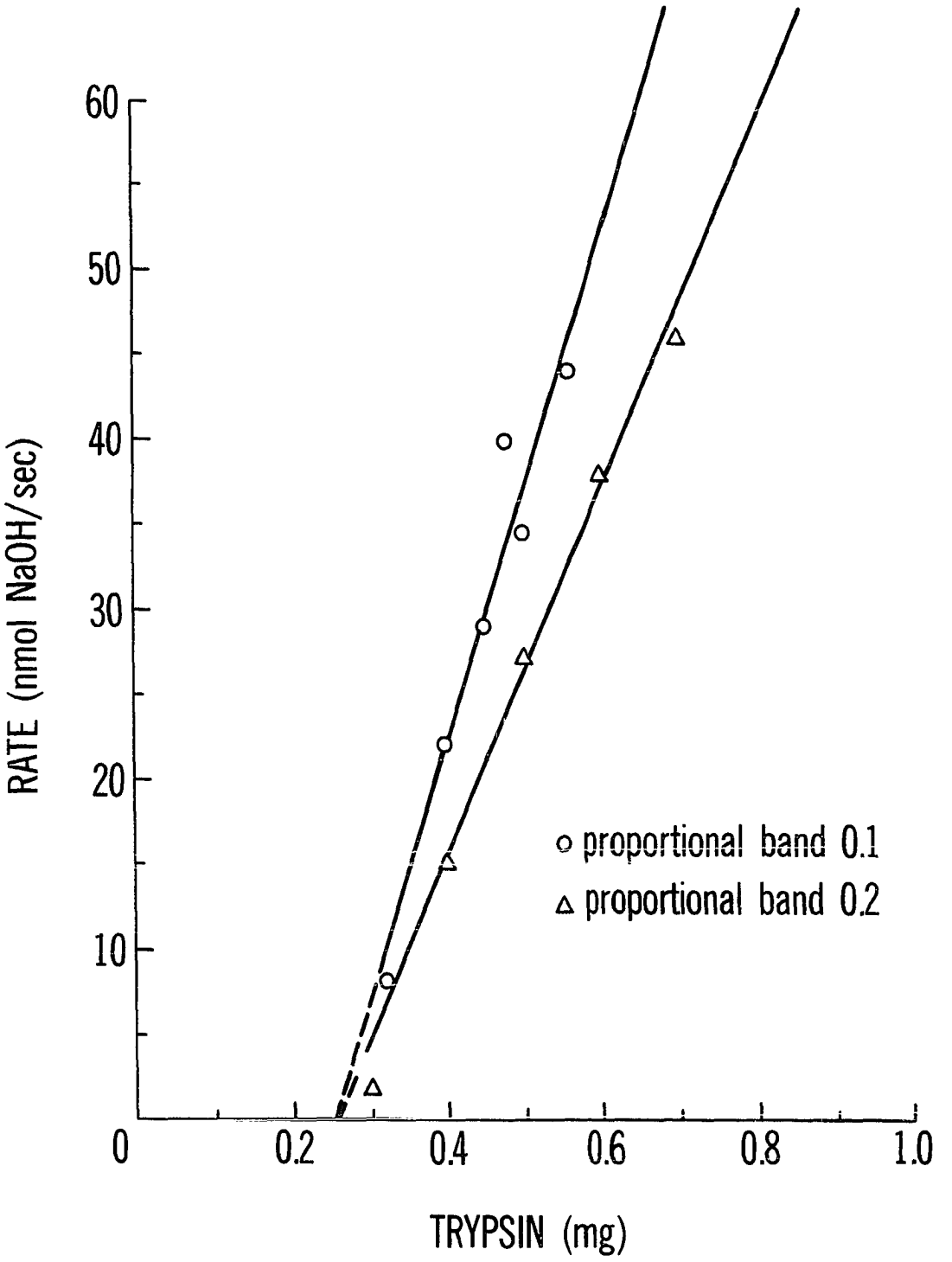
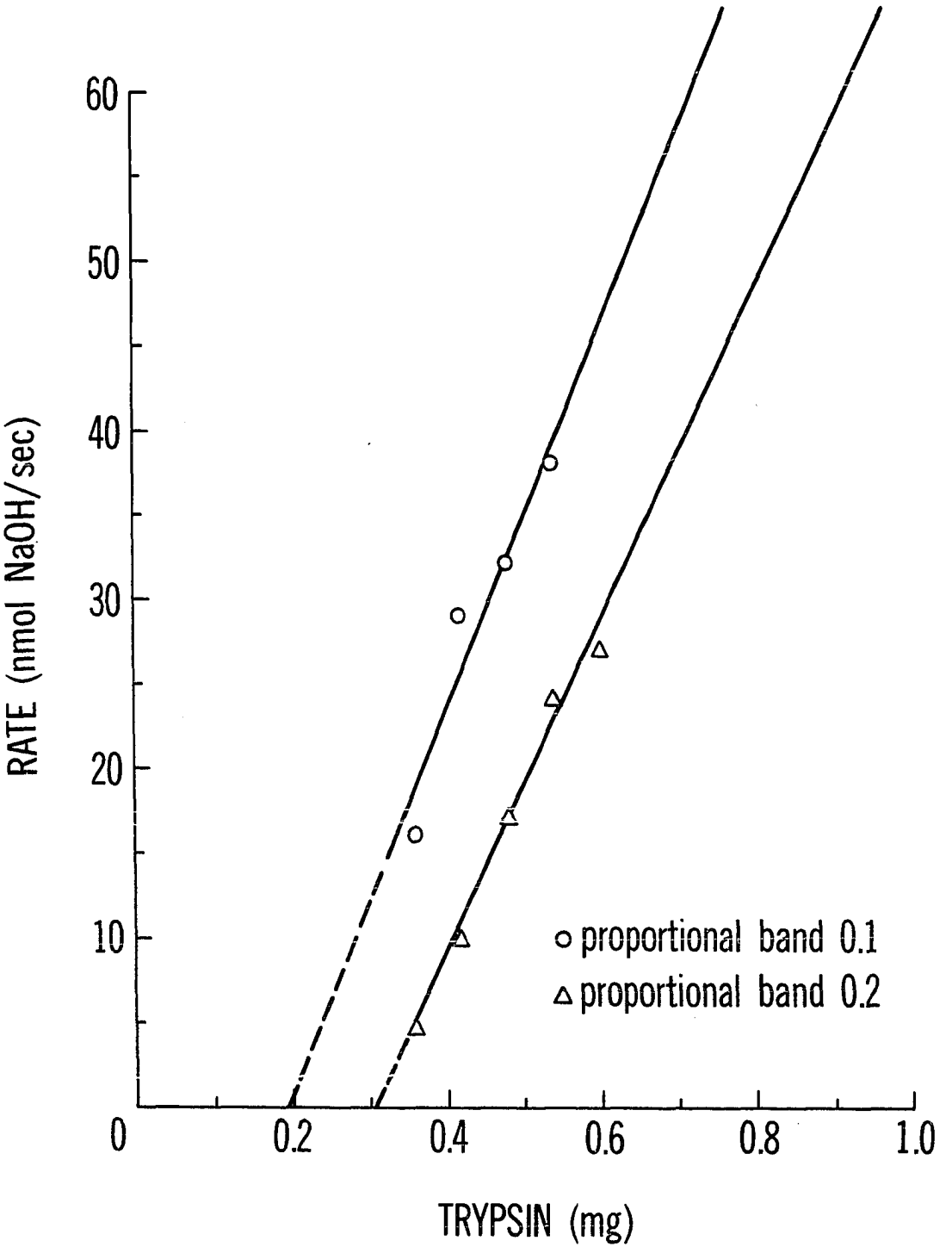

60
50
40
30
20
10
0
0.2
0.4
0.6
0.8
1.0
RATE (nmol NaOH/sec)
TRYPSIN (mg)
o proportional band 0.1
Δ proportional band 0.2

Figure 9. Effect of the proportional band setting on initial rates of tryptic hydrolysis of sample B (1.2 g/100 ml)

The instrumental parameters were: 25°C, pH 9, 0.0147 N NaOH, speed 8


60
50
40
30
20
10
0
0.2
0.4
0.6
0.8
1.0
RATE (nmol NaOH/sec)
TRYPSIN (mg)
o proportional band 0.1
Δ proportional band 0.2

During pH stat recording, proportional control is used to prevent "overshooting" of the set endpoint. If the proportional band is set at 0.1, the autoburette motor will run at maximum speed until the working pH is within 0.1 pH unit of the set endpoint. It will run discontinuously with smaller and smaller current impulses as the pH comes closer to the endpoint. However, the set endpoint is never reached in practice. The working pH deviates more from the set endpoint as the rate of the reaction studied increases. For example, in Figures 8 and 9, the working pH may be slightly lower at the enzyme concentration 0.7 mg, than at 0.4 mg.

At the proportional band setting 0.2, the autoburette motor will run continuously until the working pH is within 0.2 pH unit of the set endpoint, then it will run discontinuously. The working pH deviates more from the set endpoint (pH 9.0) as the proportional band setting increases, especially during fast reactions. At the proportional band settings 0.1 and 0.2, the working pH may be as low as 8.9 and 8.8, respectively, when the reaction rate is very fast.

It is possible to calculate how much of the rate difference between the proportional band settings of 0.1 and 0.2 might be due to a shift in the working pH and hydrogen ion dissociation of the newly formed amino groups. The Henderson-Hasselbalch equation can be used to calculate the number of

hydrogen ions released from a specific number of peptide bonds hydrolyzed at different pH values. For example, if 11 nmol of peptide bonds were hydrolyzed per second, 9.5 nmol of hydrogen ions per second would be released from the newly formed amino groups at pH 8.8 and 9.7 nmol of hydrogen ions per second would be released at pH 8.9, assuming the average pK value of the newly formed amino groups is 8. The ratio of these theoretical rates (9.5/9.7) is 0.98. The ratio of actual rates of sample A with 0.5 mg trypsin at the two proportional band settings (26/37) is 0.70. If a shift in the working pH and hydrogen ion dissociation accounted for all of the difference due to the proportional band setting, the ratio should not be less than 0.98. Hence only a small part of the rate difference can be attributed to these factors. Another factor that would influence the measured hydrolysis rates is a change in buffering due to preexisting groups (such as the phenolic group of tyrosine, the sulfhydryl group of cysteine and the ε-amino group of lysine).

Making a ratio of rates for the proportional band settings 0.1 and 0.2 at the same enzyme concentration is deceiving, unless the x-axis intercepts are the same. For example, in sample B, the ratio changes significantly with enzyme concentration (Figure 9). However, if the intercepts were shifted together, the ratios obtained for samples A and B would be similar.

At either proportional band setting, a linear relationship exists between the rate and enzyme concentration. However, use of a lower proportional band setting (0.1) would keep the working pH closer to the endpoint (pH 9.0).

Speed Figures 10 and 11 show the effects of speed on the initial reaction rates of tryptic hydrolysis of samples A and B. The autoburette has several speed settings to control the motor speed and the rate of titrant flow. The speed (speed x 10 = percent of burette capacity per minute) should be at least slightly higher than the maximum volume of titrant per minute required to fulfill the basic conditions of constant pH. If the speed is too low, the autoburette motor will run continuously, and the working pH will deviate significantly from the set endpoint. Using a speed setting that is much higher than that required to meet the maximum requirements of the process will result in reduced quality of recording. Pronounced steps will occur in the curve, due to overshooting of the endpoint.

At speed 16, the initial reaction rate is faster than at speed 8 for both samples A and B (Figures 10,11). The working pH of the system is kept closer to the set endpoint at the higher speed, under these conditions. At the slower rates, less difference is seen due to speed. At either speed setting, a linear relationship exists between the rate and enzyme concentration.

NaOH concentration

The effects of NaOH concentration on the initial rates of proteolysis of samples A and B can be seen in Figures 12 and 13. In general, the rates increased with NaOH concentration. At the higher NaOH concentration, the working pH is kept closer to the set endpoint, resulting in faster rates.

Figure 10. Effect of the speed setting on initial rates of tryptic hydrolysis of sample A (1.2 g/100 ml)

The experimental parameters were: 25°C, pH 9, 0.0147 N NaOH, proportional band 0.2

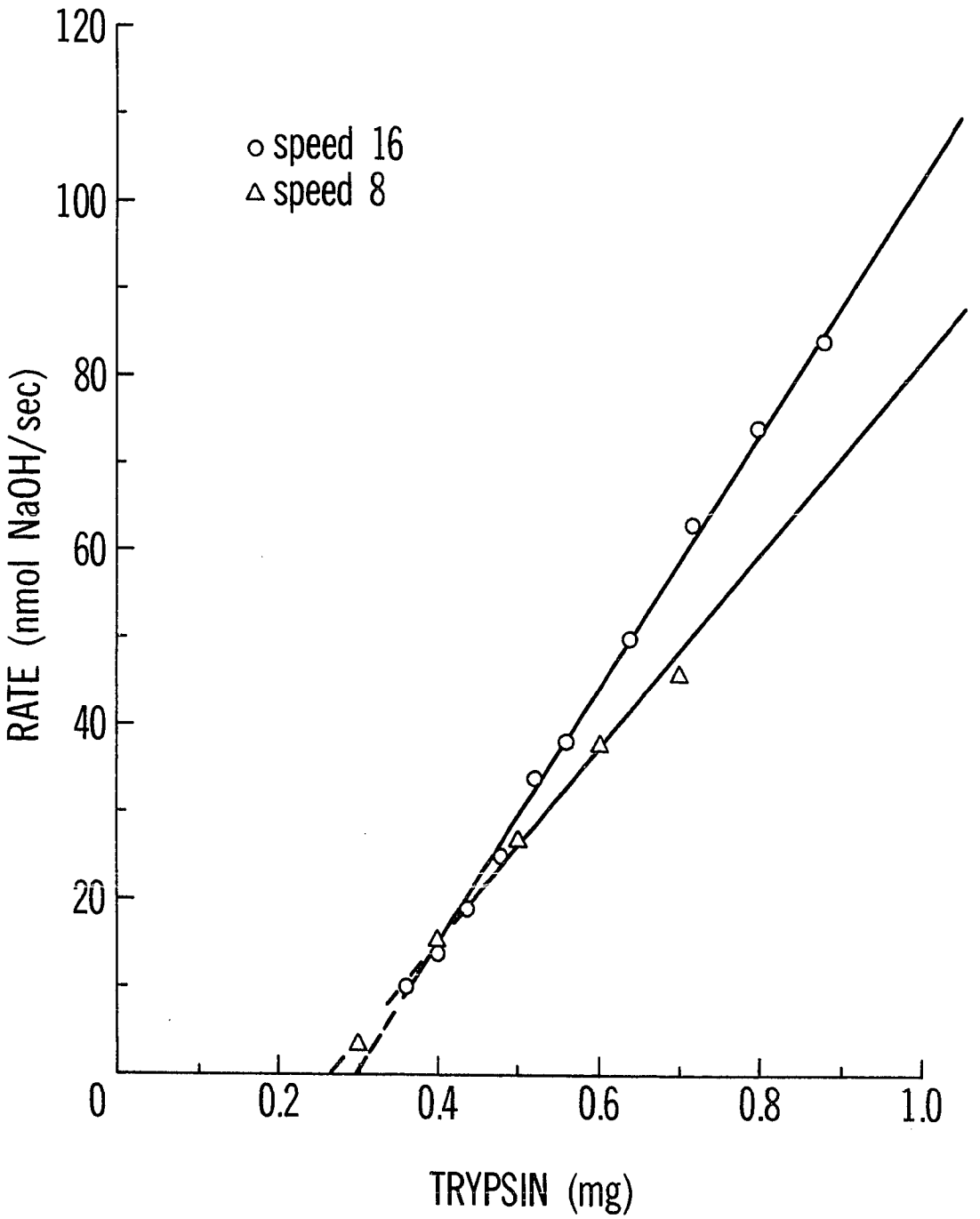

120
100
80
60
40
20
0
o speed 16
Δ speed 8
RATE (nmol NaOH/sec)
0.2
0.4
0.6
0.8
1.0
TRYPSIN (mg)

Figure 11. Effect of the speed setting on initial rates of tryptic hydrolysis of sample B (1.2 g/100 ml)

The experimental parameters were: 25° C, pH 9, 0.0147 N NaOH, proportional band 0.2

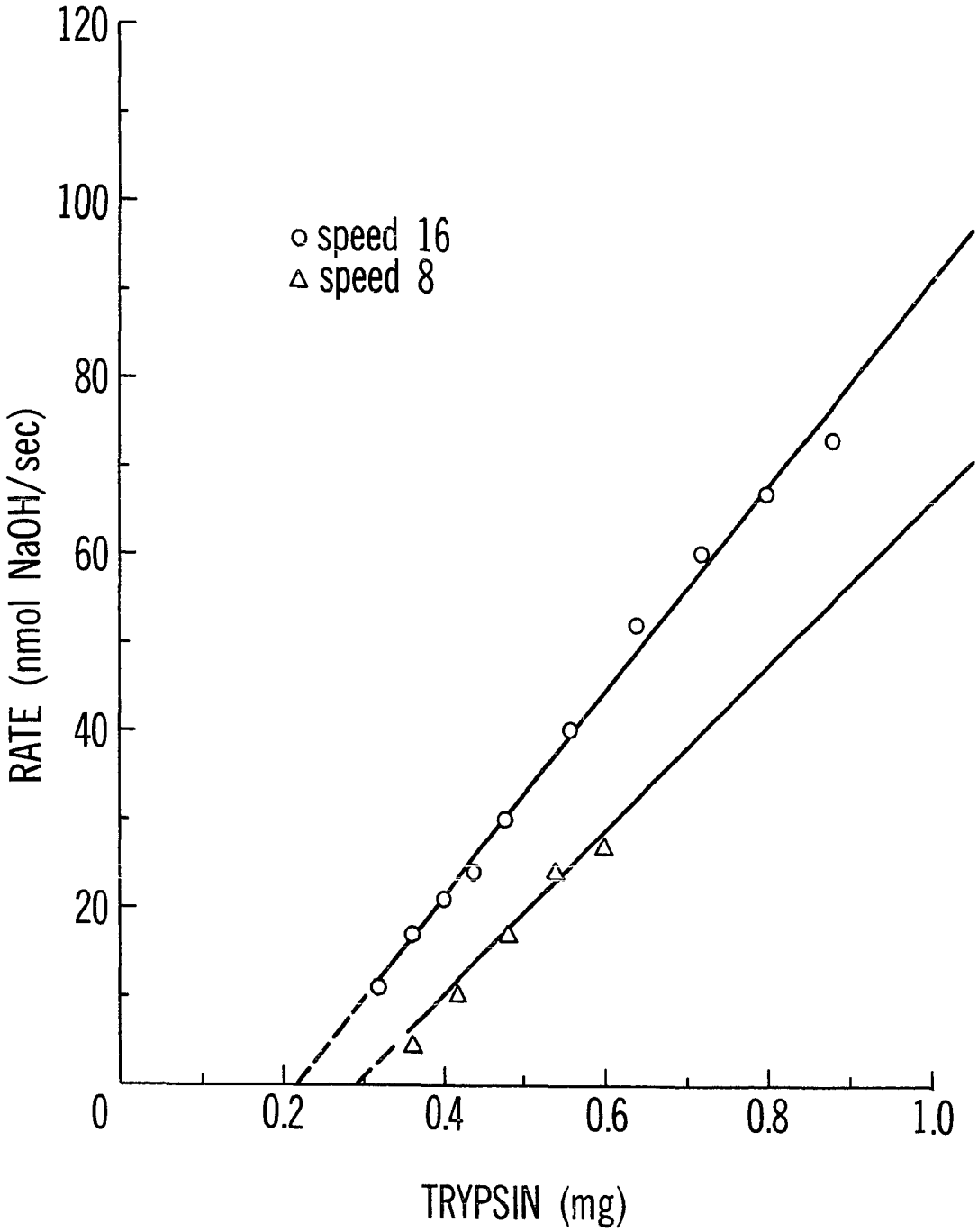

120
100
80
60
40
20
0
0.2
0.4
0.6
0.8
1.0
RATE (nmol NaOH/sec)
TRYPSIN (mg)
o speed 16
Δ speed 8

Figure 12. Effect of NaOH concentration on initial rates of tryptic hydrolysis of sample A (1.2 g/100 ml)

The experimental parameters were: 25°C, pH 9, proportional band 0.1, speed 8

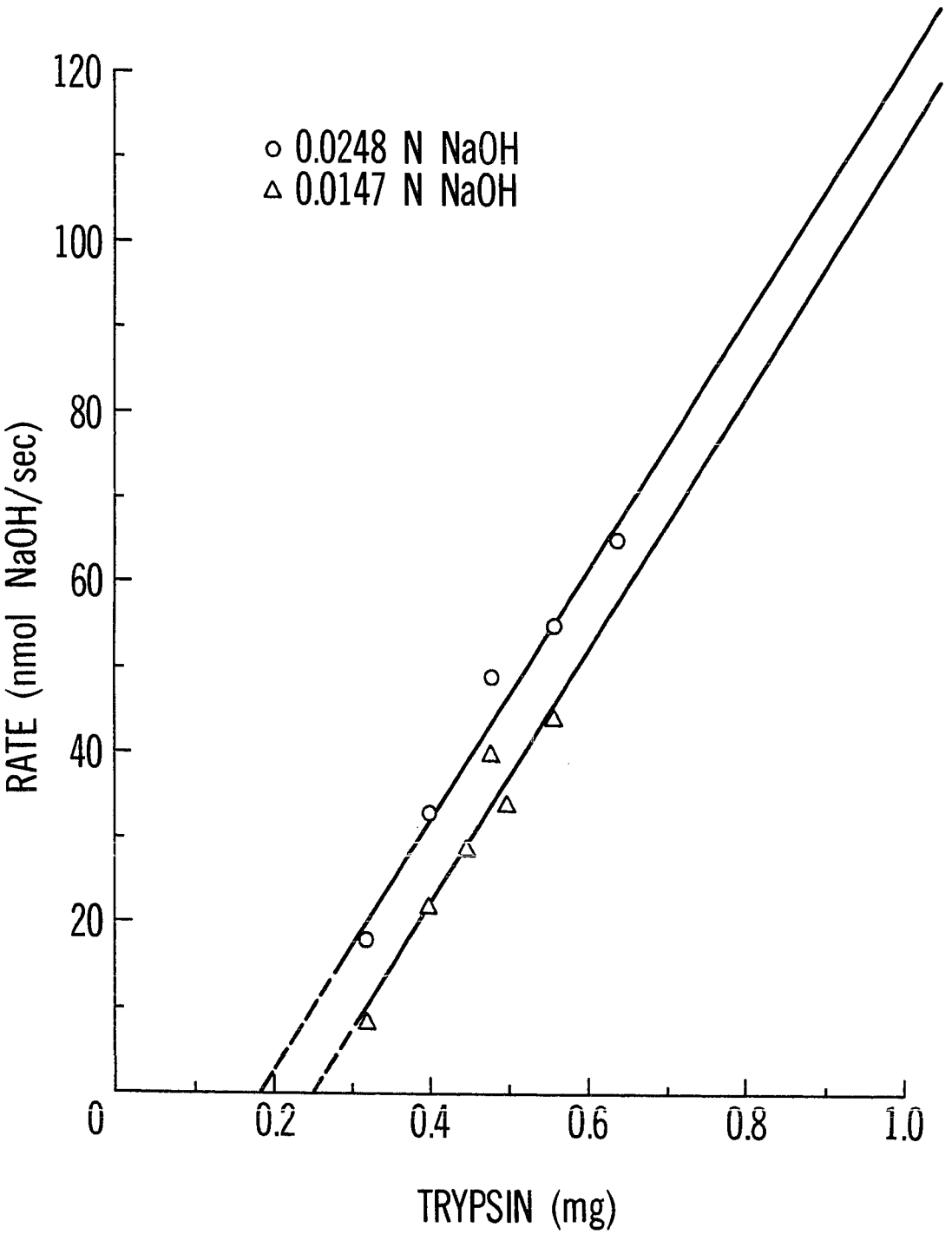
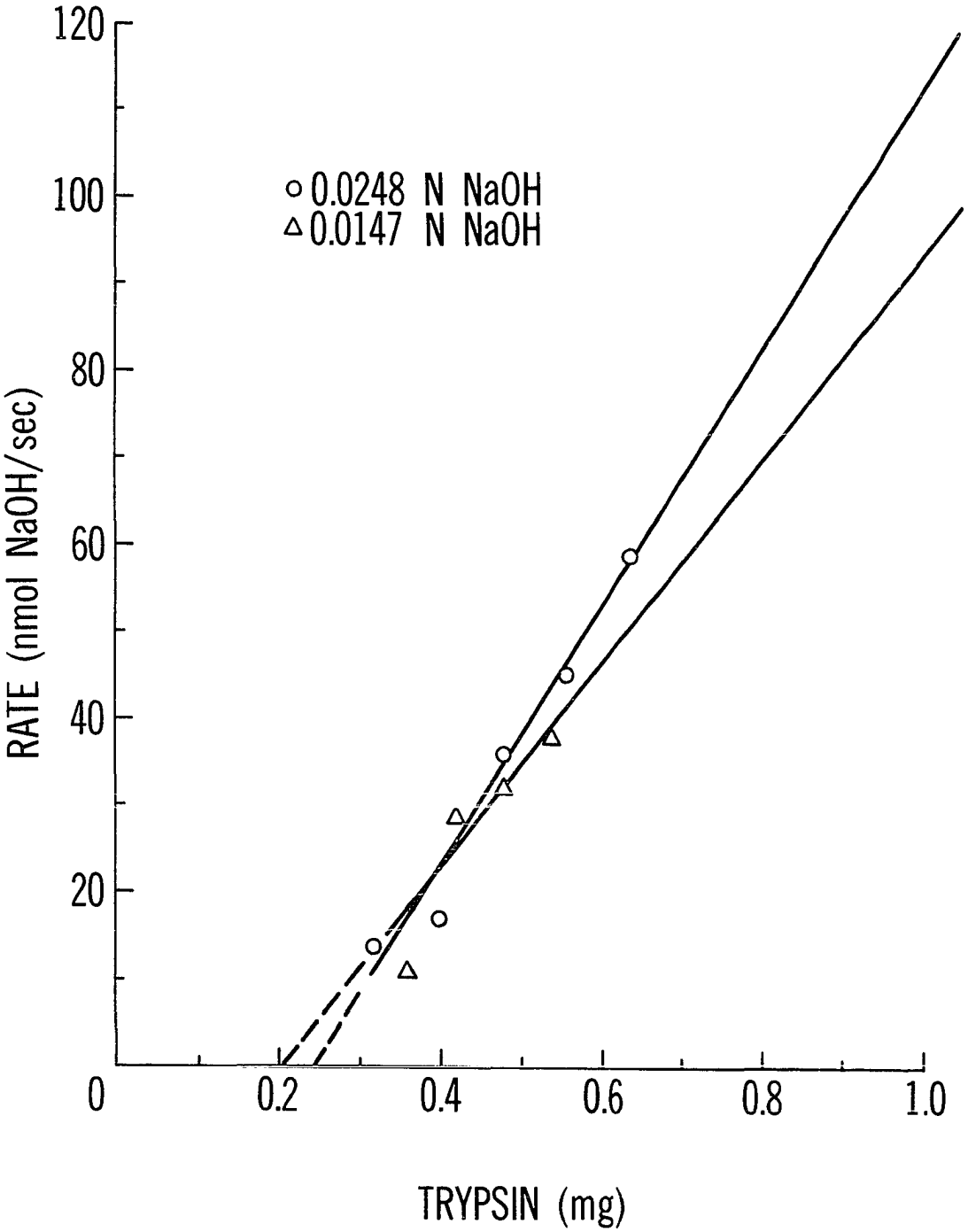

120
100
80
60
40
20
0
0.2
0.4
0.6
0.8
1.0
○ 0.0248 N NaOH
△ 0.0147 N NaOH
RATE (nmol NaOH/sec)
TRYPSIN (mg)

Figure 13. Effect of NaOH concentration on initial rates of tryptic hydrolysis of sample B (1.2 g/100 ml)

The experimental parameters were: 25° C, pH 9, proportional band 0.1, speed 8


120
100
80
60
40
20
0
0.2
0.4
0.6
0.8
1.0
○ 0.0248 N NaOH
△ 0.0147 N NaOH
RATE (nmol NaOH/sec)
TRYPSIN (mg)

## Tryptic Hydrolysis of Soy Proteins

### Chemical analyses of soy flours

The composition of the soy flour samples were very similar (Table 1). The PDI values and the trypsin inhibitor activity indicate the amount of heat processing the flours have received. Comparison of the data to literature values indicated that samples A and B were moderately heated, while sample C received a minimum of heat treatment (Kakade et al., 1969; Kakade et al., 1974; Rackis et al., 1974; Rackis and McGhee, 1975).

Table 1. Chemical analyses of soy flour

| Sample | Moisture (%) | Lipid[a] (%) | Protein[a] (%) | PDI | TUI/mg flour |
|---|---|---|---|---|---|
| A | 4.91 | 0.56 | 51.92 | 23.5 | 23.0 |
| B | 5.57 | 0.78 | 51.42 | 54.8 | 23.1 |
| C | 5.12 | 0.48 | 51.92 | 86.7 | 99.0 |

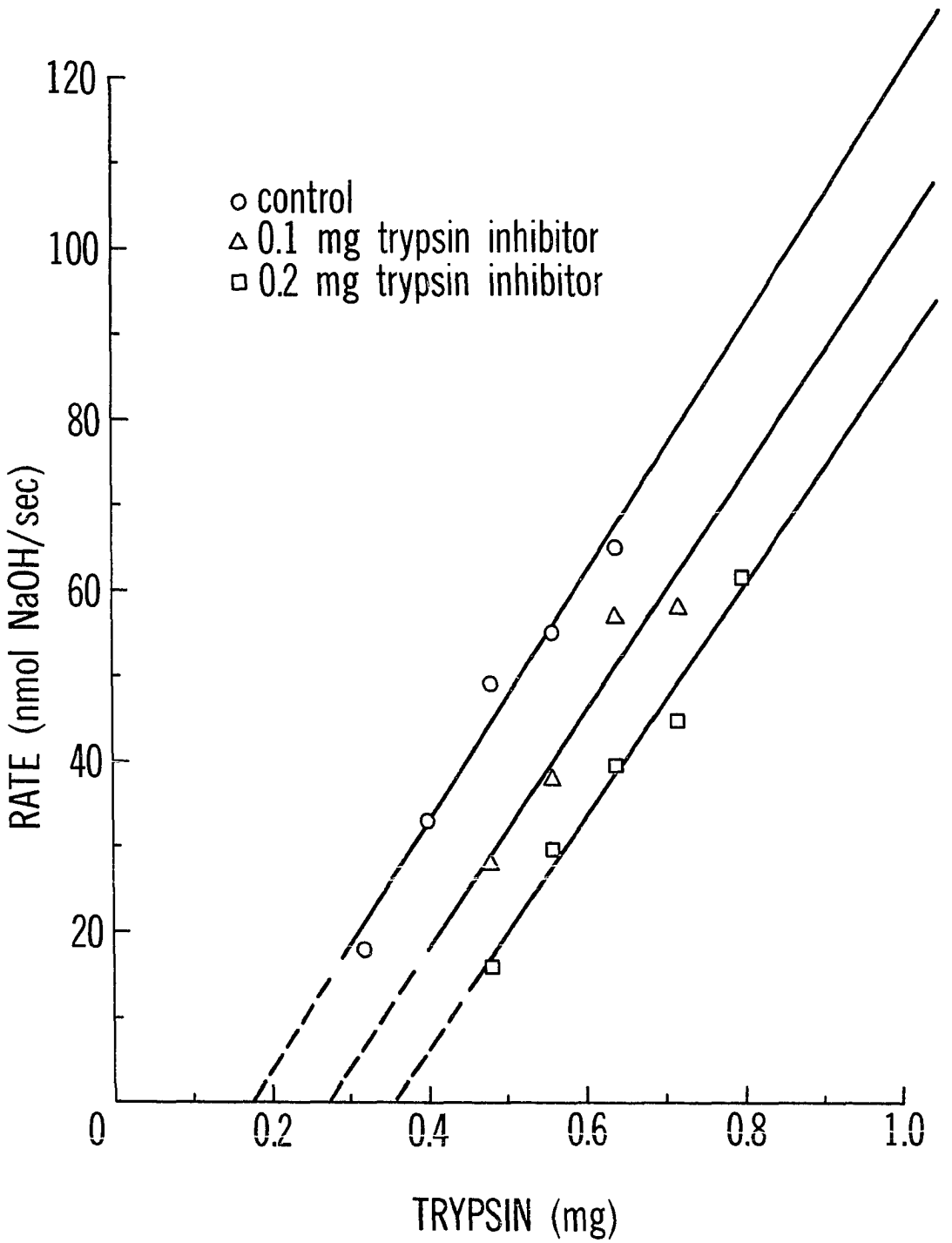
[a]Wet weight basis.

### Effect of added soybean trypsin inhibitor on initial reaction rates

The effects of added soybean trypsin inhibitor (Kunitz) on the initial rates of proteolysis of sample A are shown in Figure 14. A shift in the intercept occurred

Figure 14. Effect of added soybean trypsin inhibitor on initial rates of tryptic hydrolysis of sample A (1.2 g/100 ml)

The experimental parameters were: 25°C, pH 9, 0.0248 N NaOH, proportional band 0.1, speed 8


120
100
80
60
40
20
0
0.2
0.4
0.6
0.8
1.0
RATE (nmol NaOH/sec)
TRYPSIN (mg)
o control
Δ 0.1 mg trypsin inhibitor
□ 0.2 mg trypsin inhibitor

when 0.1 mg or 0.2 mg of trypsin inhibitor was added to the reaction mixture. The manufacturer specified that 1 mg of the trypsin inhibitor would inhibit 1.5 mg of trypsin with 10,000 BAEE units/mg protein. Assuming that 1 mg of the trypsin inhibitor would inhibit 1.05 mg trypsin with 14,350 BAEE units/mg protein, a one to one interaction between the trypsin inhibitor and trypsin should occur. The actual shifts in the intercept shown in Figure 14 agree with this assumption. For example, the addition of 0.1 mg trypsin inhibitor results in a shift in the intercept that corresponds to 0.1 mg trypsin. The shifts in the intercept may be due to the formation of a trypsin-inhibitor complex, that is devoid of either tryptic or inhibitory activity (Laskowski and Laskowski, 1954). The affinity of trypsin for the trypsin inhibitor is much greater than that for natural substrates, about $10^9$ compared to $10^2$ (Green, 1953). The added trypsin inhibitor may bind trypsin stoichiometrically, requiring additional trypsin to achieve the same rate of hydrolysis of the soy protein. Stoichiometric inhibition indicates that the enzyme-inhibitor complex has a very high association constant and does not exhibit enzymatic activity (Frattali, 1969). The slopes of the reaction rate versus trypsin concentration curves are parallel, which suggests that the slopes of the curves are due to the influence of denaturation on tryptic hydrolysis rates. The shifts in the intercepts of the curves reflect the interaction of trypsin and the trypsin inhibitor.

Comparison of soy flours

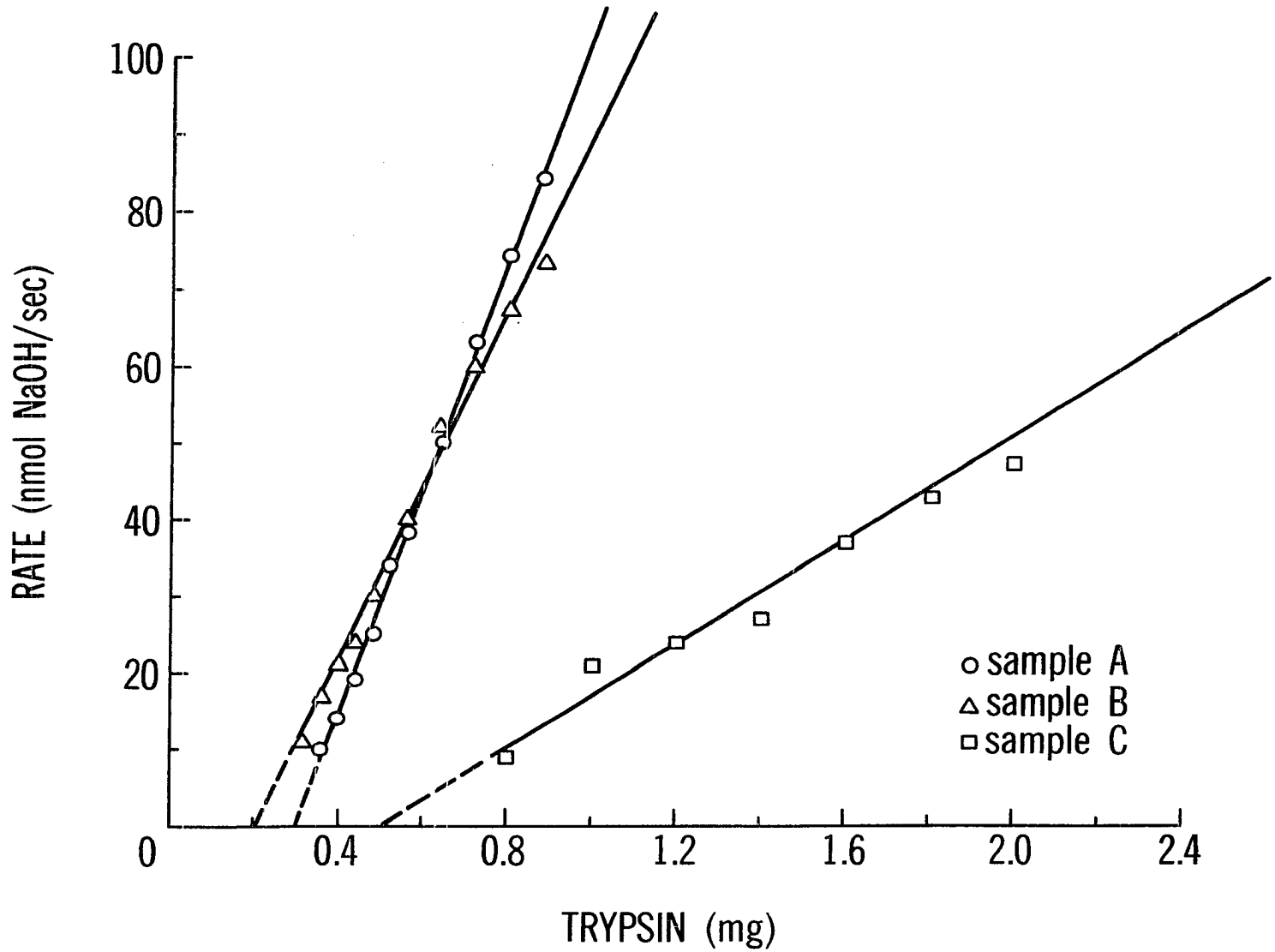
Heat increases the digestibility of soy protein products by inactivating the trypsin inhibitors and by denaturing the protein. Changes in tryptic hydrolysis of

soy flour, monitored by the pH stat, should reflect the amount of heat treatment the flours have received. A comparison of the initial rates of hydrolysis of samples A, B and C versus trypsin concentration is shown in Figure 15. The slopes of the curves are significantly different at the 1% confidence level. The slopes may indicate the amount of protein denaturation that occurred in the samples. An increase in the slopes occurred with increasing heat treatment. The shifts in the intercepts may reflect the interaction of the trypsin inhibitors and trypsin. However, the shifts in the intercepts are not proportional to the amount of trypsin inhibitor activity present in the samples. Sample C contains about 4 times as much trypsin inhibitor activity as samples A or B; however, the shift in the intercept of sample C is only about 2 1/2 times as much (Table 1, Figure 15).

Several different trypsin inhibitors are present in soy protein products. Although the Kunitz inhibitor seems to shift the intercept proportionally to the amount added (Figure 14), the other trypsin inhibitors may interact with trypsin differently. The two main trypsin inhibitors present in soy protein are the Kunitz and the Bowman-Birk inhibitors. The Kunitz inhibitor reacts stoichiometrically with trypsin to form an inactive complex (Laskowski and Laskowski, 1954). However, no direct proportionality exists between the amount of Bowman-Birk inhibitor added and the amount of trypsin inhibited (Frattali, 1969). A near linear relationship between the amount of trypsin inhibited for a given amount of Bowman-Birk inhibitor has been reported up to about 70% inhibition (Frattali,

Figure 15. Effect of heat processing on initial rates of tryptic hydrolysis of soy flour (1.2 g/100 ml)

The experimental parameters were: 25°C, pH 9, 0.0147 N NaOH, proportional band 0.2, speed 16


100
80
60
40
20
0
RATE (nmol NaOH/sec)
0.4
0.8
1.2
1.6
2.0
2.4
TRYPSIN (mg)
sample A
sample B
sample C

1969). However, beyond this point the relationship deteriorated and inhibition was incomplete even in the presence of excess inhibitor.

The stability of the two inhibitors to heat treatment also differs. The Bowman-Birk inhibitor is more stable than the Kunitz inhibitor (Birk, 1961). Since samples A and B received more heat treatment than sample C, they may contain a higher proportion of the Bowman-Birk inhibitor. Although the shifts in the intercepts cannot be satisfactorily explained, an interaction between the trypsin inhibitors and trypsin is probably involved.

The comparison of the initial rates of soy flour hydrolysis in Figure 15 indicates that the pH stat method can detect differences due to heat processing. There is a greater difference between samples B (PDI, 54.8) and C (PDI, 86.7) than between samples A (PDI, 23.5) and B (PDI, 54.8). Hsu et al. (1977) also found this trend to be true using an in vitro technique based on a pH decrease after 10 minutes of hydrolysis. The digestibility values for soy flour samples with PDI values of 20, 70 and 90 were 82.11, 77.10 and 72.80 percent, respectively. PER and nitrogen digestibility values from rat bioassays also follow this trend (Rackis and McGhee, 1975). A rapid increase in the digestibility of soy flour occurs initially with heat treatment, followed by a slower increase. From these comparisons, I would predict that the initial rates of proteolysis of a soy flour with a PDI of 70 would fall about halfway in between samples B and C.

These preliminary experiments indicate that the pH stat method has good

potential for evaluating heat processing of soy flours. This method is sensitive enough to detect the effects of processing and the presence of trypsin inhibitors. Even though the instrumental parameters as well as the substrate concentration affect the reaction rates, using a set of standard conditions would minimize these effects between samples. I would suggest using the following conditions for proteolysis of soy flour: temperature, 25° C; endpoint, pH 9.0; proportional band, 0.1; speed, 16; NaOH, 0.15 N; and substrate concentration, 1.2 g/100 ml. In future work, a set of standard rate curves for a wide range of soy flour samples with different heat treatments should be made. Once the relationship between heat treatment and reaction rates has been established, this procedure would be rapid and simple to perform. It appears that this method is most sensitive to soy flour samples with PDI values above 55. In this range, an initial reaction rate determined at a single enzyme concentration may be sufficient for evaluating heat processing. However, samples with PDI values below 55 may have to be evaluated by differences in the slopes of the reaction rate versus enzyme concentration curves. Although the slopes increase with heat treatment, a comparison of samples A and B in Figure 15 indicates that in some cases (between PDI 23.5 and 54.8), even differences in slopes may not be sufficient for evaluating heat processing.

# SUMMARY AND CONCLUSIONS

This study was conducted to evaluate the effects of heat processing on soy proteins, using a pH stat to monitor tryptic hydrolysis. Heat increases the digestibility of soy proteins by inactivating the trypsin inhibitors and by denaturing the protein. Changes in tryptic hydrolysis of soy flour should reflect the amount of heat treatment the flours have received. Proteolysis of soy flour was followed by a pH stat at pH 9 and 25° C.

Initial reaction rates increased as the speed setting and NaOH concentration increased, and as the proportional band setting decreased. Under these conditions, the working pH of the system remained closer to the set endpoint (pH 9.0). An increase in the working pH results in an increase in free hydrogen ions. However, actual calculations indicated that only a small part of these rate differences could be attributed to a change in the working pH.

Initial reaction rates increased as the substrate concentration decreased. Trypsin inhibitor activity and the buffering capacity of the protein are factors involved in this unusual relationship.

A linear relationship exists between the enzyme concentration and the initial reaction rates. However, the intercept of the line does not go through zero. The shift in the intercept may be due to the interaction of trypsin with the trypsin inhibitors present in the soy flours. Addition of Kunitz trypsin inhibitor to sample A

resulted in a stoichiometric shift in the intercept. However, when comparing soy flour samples with different heat treatments, the shifts observed in the intercepts were not proportional to the amount of trypsin inhibitor activity initially present. When Kunitz trypsin inhibitor was added to sample A, the slopes of the reaction rates versus trypsin concentration curves were parallel. Although the slopes did not change due to added trypsin inhibitor, they increased with heat treatment. The increased slopes are probably due to protein denaturation and increased susceptibility to trypsin.

The pH stat method is sensitive enough to detect the effects of processing and the presence of trypsin inhibitors. Standard conditions should be used to minimize the effects of instrumental parameters, NaOH concentration and substrate concentration. Once the different rate curves have been established in relation to heat treatment, this method would be rapid and simple to perform, and should provide useful information about the nutritive value of soy flour.

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