LEVELS OF TRYPSIN INHIBITORS IN SOY-BASED FOODS AND MODULATION OF THEIR ANTINUTRITIONAL EFFECTS BY DIETARY AMINO ACIDS

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ABSTRACT

Doctor of Philosophy Animal Science (Nutrition)

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Levels of soybean trypsin inhibitors (SBTI) in soy-based infant formulas were measured and found to range from 3 to 28% of the activity measured in raw soybeans, with higher activity usually present in ready-to-feed compared to concentrate or powder formulations. Experiments were conducted to examine the influence of dietary SBTI on growth, serum enzyme, lipid and free amino acids and hepatic S-adenosylmethionine (SAM) status in weanling male rats. Diets containing graded amounts of SBTI were fed with and without the methionine antagonist ethionine. Changes in growth, serum enzymes, lipid and amino acid parameters in rats fed SBTI or ethionine indicated lipotrope deficit and compromise of the transsulfuration pathway. The combination of SBTI and ethionine exacerbated many of the symptoms and methyl donor deficit was indicated by hepatic SAM status. Methionine supplementation of SBTI + ethionine diets was beneficial at moderating changes while cysteine supplementation was not.

Resumé

Docteur en philosophie Science Animal (Nutrition)

Robert William Peace

TENEUR EN INHIBITEURS DE TRYPSINE DES ALIMENTS
A BASE DE SOYA ET MODULATION DE LEURS EFFETS ANTINUTRITIFS
PARS DES ACIDES AMINES

La teneur en inhibiteurs de trypsine de soya (SBTI) des formules de soya pour nourrissons a été mesurée. Il a été observé que l'activité SBTI variait de 3 à 28% de celle de la fèvre crue; les formules prêts-a-servir ont montré une plus forte activité que les concentrés ou les poudres. L'effet des SBTI alimentaires sur la croissance, les concentrations sériques des transaminases, lipides et acides aminés, et sur les concentrations hépatiques de la S-Adénosylméthionine (SAM) a été étudié chez le rat sevré. Des régimes contenant différents taux de SBTI ont été administrés avec ou sans éthionine, un antagoniste métabolique de la méthionine. Les changements du taux de croissance, et des transaminases, lipides, et acides aminés sériques chez les rats sous régime SBTI ou éthionine ont indiqué une carence lipotropique et une dimunition de la voie de transsulfuration. La combinaison SBTI et éthionine a exacerbé plusieurs des symptômes et le status de la SAM hépatique a révélé une manque de groupements méthyl. L'ajout de méthionine, mais non de cystéine aux régimes SBTI + éthionine a atténué ces effets négatifs.

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CLAIM OF ORIGINALITY AND CONTRIBUTION TO KNOWLEDGE

To the author's knowledge, the following aspects of this study constitute an original contribution to research and expand knowledge of the role of soybean trypsin inhibitors (SBTI) and methods to assay for their effects.

1. The development of methods for the determination of trypsin inhibitors in soy-based infant formulas. This included determination of the optimal extraction buffer to be used, the utilization of temperature controlled lyophilization to concentrate liquid formula preparations and low temperature fat extraction to remove potential lipid interferences with minimal destruction of heat labile trypsin inhibitors.

2. The optimization of HPLC methods for rapid measurement of S-adenosyl-derivatives of methionine and ethionine in hepatic tissues of the rat using Waters SCX-cation exchange columns and radial compression technology.

3. The demonstration that relatively high levels of trypsin inhibitors can occur in modern preparations of soy-based infant formulas.

4. The finding of the following significant effects of dietary intakes of the carcinogenic antilipotrope DL-ethionine in young rats:

a) The observation that dietary DL-ethionine combined with soybean trypsin inhibitors (SBTI) when fed to weanling rats, resulted in increased serum levels of total free essential amino acids and in paricular threonine and in the accumulation of methionine sulfoxide, the primary oxidation product of methionine.

b) The observation of decreased growth and elevated serum urea nitrogen in response to dietary ethionine in young rats, that combined with decrease in serum free methionine and accumulation of total essential amino acids, were suggestive of decreased protein synthesis and blockage in availability or normal metabolic utilization of methionine. This was further

supported by accumulation of serum free serine, a substrate of the transsulfuration pathway of methionine metabolism and of the serine precursor glycine.

c) The observation of elevation of serum free taurine in response to dietary ethionine that likely resulted not from increased transsulfuration, but rather decreased taurine utilization in bile acid conjugation due to the antilipotropic effects of dietary ethionine and the accumulation of hepatic lipids.

d) The observation of increased serum levels of serum glutamate transaminase (SGPT) and alkaline phosphatase (indicators of liver cell damage) in young rats fed ethionine and SBTI High serum concentrations of these parameters, and of serum urea nitrogen were moderated or normalized by dietary methionine supplementation or by decreased dietary levels of SBTI.

5. The finding, in young rats fed diets theoretically adequate in dietary lipotropes but containing SBTI, of several biochemical changes that were similar to those induced by the antilipotrope ethionine. These included:

a) Changes in serum clinical chemistry parameters including decreases in total cholesterol, increases in triglycerides, and increases in serum glutamate pyruvate transaminase which suggested similiar antilipotropic effects of SBTI and ethionine.

b) Changes in serum levels of free amino acids that were suggestive of lipotrope deficit, impairment in the transsulfuration pathway of methionine metabolism and decrease in protein synthesis due to dietary SBTI. Decreased serum free methionine, increased total essential amino acids and increased threonine, as well as increases in the transsulfuration substrate serine and its precursors glycine and alanine and decreases in the transsulfuration end-product taurine, occurred with dietary intakes of SBTI.

c) Ethionine appeared to be a more potent antilipotrope or effector of the various clinical chemistry and serum amino acid changes than were dietary SBTI. In some instances,

combination of ethionine and SBTI increased the antilipotropic symptoms and in many cases the effects of both factors were prolonged although somewhat moderated over time. This reflected both an ongoing antilipotropic influence of the two factors as well as decreased sulfur amino acid requirements of the older rats.

6. For the first time, increases in hepatic concentrations of S-adenosylmethionine (SAM), decrease in ratio of S-adenosylmethionine/S-adenosylhomocysteine (SAM/SAH) and increase in ratio of S-adenosylethionine/S-adenosymethionine (SAE/SAM) in response to dietary SBTI and combination of SBTI and ethionine were detected. These were indicative of reduced availability of methyl donor groups and compromise of the transsmethylation-transsulfuration pathway of methionine metabolism. Reduced availability of methyl donor groups and deficits in the transmethylation-transsulfuration pathway of methionine metabolism are likely common mechanisms of the antilipotropic and carcinogenic effects of ethionine and soybean trypsin inhibitors.

7. Renal calcinosis was observed in rats fed SBTI and high levels of ethionine. Supplementary methionine appeared to minimize the incidence of renal calcinosis whereas supplementary cysteine was less effective.

8. The relative efficacy of dietary supplements of L-methionine and L-cystiene at overcoming the antilipotropic and negative methyl donor influences of dietary ethionine and SBTI in young rats was determined. Supplementary methionine increased hepatic ratios of SAM/SAH and decreased ratios of SAE/SAM. It also decreased serum levels of the essential amino acids threonine and lysine and of the non essential transsulfuration substrate serine (and the serine precursors glycine and alanine) that were elevated in response to high dietary SBTI and ethionine. L-cysteine proved largely ineffective at increasing SAM/SAH or decreasing SAE/SAM in the presence of SBTI and high ethionine. It also was not effective at overcoming

the accumulation of serine and its precursors glycine and alanine that occurred in response to dietary SBTI and ethionine.

9. The relative ineffectiveness of cysteine likely resulted from the relatively high ratio of cysteine to methionine in the soy protein fed and has important implications in terms of the correct ratio of supplementary sulfur amino acids to be used in supplementation of soy-based infant formulas. The effectiveness of supplementary methionine at moderating several of the antilipotropic effects of SBTI and ethionine suggest preference for its use as a sulfur amino acid supplement of soy-based infant formulas. The elevation of serum free taurine in response to cysteine suggests that cysteine supplementation of infant formulas in gerneral, rather than taurine supplementation, may be adequate to protect from any potential immaturity of transsulfuration-mediated taurine synthesis.

This thesis is presented as a series of 5 papers corresponding to Sections III.1 to III.5. McGill University Guidelines Concerning Thesis Preparation state : "While the inclusion of manuscripts co-authored by the Candidate and others is not prohibited by McGill, the Candidate is warned to make an explicit statement on who contributed to such work and to what extent, and Supervisors and others will have to bear witness to the accuracy of such claims before the Oral Committee. It should be noted that the task of the External Examiner is made much more difficult in such cases, and it is in the Candidate's interest to make authorship responsibilities perfectly clear."

Section III.2 has has been accepted for publication in the journal Nutrition Research, authored by Robert W. Peace, G. Sarwar and S.P. Touchburn, and H.G. Botting. Section III.1, authored by R.W. Peace, G. Sarwar, S.P. Touchburn and Section III.3 authored by R. W. Peace, G. Sarwar, H.G. Botting and S.P. Touchburn have been submitted for publication. Drs. Sarwar and Touchburn were the student's co-supervisors. Dr. J. Campbell, Pathologist, Toxicology Research Division, Food Directorate, undertook a histological assessment of tissues. Mr. Herb Botting and M. Jacques Matte, laboratory technicians, assisted in tissue sampling and Mr. Botting provided valuable guidance in HPLC techniques. Mr. Matte assisted in fat analyses and clinical chemistry measurements. Sections III.4 and III.5 will be published with the student's co-supervisors as co-authors.

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ABBREVIATIONS AND DEFINITIONS

Amino Acids

Arg	- arginine	α-Aba	- α-aminobutyric acid
His	- histidine	Ala	- alanine
Ile	- isoleucine	Asp	- aspartic acid
Leu	- leucine	Glu	- glutamic acid
Met	- methionine	Pro	- proline
Cys	- cystine	Ser	- serine
Phe	- phenylalanine	Tau	- taurine
Tyr	- tyrosine	Cit	- citrulline
Thr	- threonine	Orn	- ornithine
Trp	- tryptophan	Val	- valine
Gly	- glycine	Lys	- lysine
Eth	- ethionine	Asn	- asparagine
Gln	- glutamine	Cys(e)	- cysteine

BBI - Bowman-Birk inhibitor

CCK - cholecystokinin

KTI - Kunitz inhibitor

SAA - Sulfur amino acids

SAE - S-adenosyl-L-ethionine (S-adenosylethionine)

SAH - S-adenosyl-L-homocysteine (S-adenosylhomocysteine)

SAM - S-adenosyl-L-methionine (S-adenosylmethionine)

SBTI - soybean trypsin inhibitors, a general term for both BBI and KTI

SGOT - serum glutamate oxaloacetate transaminase

SGPT - serum glutamate pyruvate transaminase

SPI - soy protein isolate (Supro 710)

For more complete definitions, references cited in the text of the thesis are recommended.

I. INTRODUCTION

Protease inhibitors are naturally occurring factors that have widespread occurrence in both the plant and animal kingdom (Xavier-Filho and Campos 1989). Soybean trypsin inhibitors (SBTI) consist of two types of plant-derived protease inhibitors that have been subject to major study because of their early discovery, their occurrence in relatively high concentrations in the seeds of a plant of major economical importance and because of their effects on animals. While SBTI have endogenous functions within the soy plant which likely include control of seed dormancy and plant development (Ashton 1976, Wilson 1980) and defense against microbial or insect attack (Green and Ryan 1972, Janzen et al. 1986), their antinutritional and physiological effects on animals that consume them are of primary importance.

SBTI cause reduced growth, decreased protein digestibility, depressed metabolizable energy, reduced fat absorption, and reduced availability of amino acids, vitamins and minerals (Rackis 1974, Rackis and Gumbmann 1981) in susceptible species and can also have an indirect but major trophic effect on the pancreas resulting in increased synthesis of proteins, phospholipids and nucleic acids, increased synthesis and secretion of proteases and hypertrophy and hyperplasia (Melmed et al. 1976, Rothman 1986).

The mechanisms underlying their influence in animals have not been fully elucidated. The formation, however, of relatively stable and inactive inhibitor-enzyme complexes within the small intestine results in decreased levels of active proteases and a loss of normal feedback inhibition on pancreatic proteolytic enzyme synthesis and secretion (Green and Lyman 1972, Schneeman and Lyman 1975). The loss of cysteine-rich proteases and the

decreased digestibility of soy protein because of complex formation and loss via the faeces decreases the availability and increases the requirement for amino acids (Rackis and Gumbmann 1981, Bender 1987).

Sulfur amino acids in particular appear to be affected because of their inherently low levels in legume protein and because of their relatively high concentration in the inactivated proteases (Bressani and Elias 1974, Liener 1976). Sulfur amino acid supplementation appears to be effective to varying degrees in different animal species in alleviating the antinutritional effects of dietary SBTI (Temler et al. 1974, Bajjalieh et al. 1980, Friedman et al. 1984).

Dietary SBTI have also been shown to potentiate the effects of weak pancreatic carcinogens such as azaserine and di(2-hydroxypropyl)nitrosamine in different animal species (Levison et al. 1979, McGuinness et al. 1984). Long-term rat feeding studies also demonstrate that SBTI present in both soy protein isolates and raw soy can induce the development of pancreatic nodular hyperplasia, acinar cell adenoma and neoplasia (McGuinness et al. 1980, Spangler et al. 1985). The underlying mechanism of these carcinogenic effects remain to be elucidated.

While SBTI are considered to be heat labile and almost completely inactivated by normal processing, residual levels may remain in soy-based products because of the need to balance SBTI destruction with the potential adverse effects of overprocessing on other nutrients (Anderson et al. 1979, Rackis et al. 1986). Humans daily consume considerable quantities of protease inhibitors from both plant and animal sources (Doell et al. 1981) and there are risk groups such as vegans, individuals on legume-based cholesterol-lowering diets, and infants fed soy-based formulas as a sole source of nutrition that may be exposed to higher amounts of SBTI than the normal population (McGuinness et al. 1984).

Levels or activities of SBTI have been measured in many foods (Doell et al. 1981), although data on soy-based infant formulas is incomplete (Theuer and Sarett 1970, Churella et al. 1976). Levels of SBTI in newer formulations and different forms of soy-based formulas have not been reported.

Dietary lipotropes (choline, methionine, vitamin B_{12}, and folate) are required for normal lipid metabolism in the body and are the main dietary precursors of methyl groups. They also contribute to the biosynthesis of membrane constituents such as phosphatidylcholine, sphingomyelin and plasmalogens and the formation of the neurotransmitter acetylcholine (Zeisel et al. 1989). Lipotrope deficiencies have been implicated in the development of different types of cancers in man and laboratory animals both in the presence and absence of chemical carcinogens (Mikol et al. 1983, Ghoshal and Farber 1984, Newberne and Conner 1986).

It has been proposed that lipotrope deficits may enhance free radical formation, decrease microsomal mixed function oxidase activity and permit nucleolar lipid peroxidation and DNA methylation changes (Rushmore et al. 1984,1986). Deficits in availability of dietary lipotropes whether induced by dietary restriction or by the use of chemicals to block or increase their utilization, can also lead to a deficiency of labile methyl groups in the body (Finkelstein et al. 1988, Henning et al. 1989).

Biological methylations are of fundamental importance in the synthesis of many important macromolecules, and are also involved in the detoxification and removal of xenobiotics (Newberne and Conner 1986). The hypomethylation of DNA has been proposed to permit inappropriate genome expression and in the case of oncogenes, transformation and cancer development (Hoffman 1984). Transmethylation and the closely connected transsulfuration pathway of methionine metabolism give rise to the activated sulfonium

compound S-adenosylmethionine (SAM), and other important sulfur amino acid derivatives. SAM serves as a source of methyl groups for various transmethylation reactions of the body and transfer of sulfur groups via transsulfuration produces amino acid derivatives such as taurine which is required for normal bile acid transport (Finkelstein 1990). Cysteine, another derivative, if supplied in the diet can partially spare dietary methionine requirements for growth (Byington 1972) and the correct ratios of methionine to cysteine (or its dimer cystine) in infant formulas are an area of active interest (Zlotkin and Anderson 1982, Fomon et al. 1986).

Methyl group deficit is characterized by decreases in the body level of SAM and decreases in the ratio of SAM/SAH (Shivapurkar and Poirier 1983, Hoffman et al 1978, Henning et al. 1989). Inadequate dietary methionine relative to rat growth requirements has been shown to result in poor growth and the serum accumulation of essential amino acids other than methionine and of non-essential amino acids related to the transsulfuration pathway (Peace et al. 1986).

The objectives of the present research are outlined as follows:

1. To determine the levels of SBTI in different formulations and preparation forms of soy-based infant formulas.

2. To examine the influence of SBTI fed in their normal dietary form (in soy protein) on weanling rats, with respect to indicators of general lipotrope or methionine adequacy including growth and clinical biochemical markers such as serum enzymes, serum lipids and serum free amino acids related to the transsulfuration pathway of methionine metabolism

3. To examine the influence of dietary SBTI on indicators of lipotrope adequacy in rats fed diets containing DL-ethionine, a known antilipotropic agent and carcinogenic antagonist of methionine.

4. To examine the influence of dietary SBTI and DL-ethionine on methyl group status of weanling rats by measuring hepatic levels of SAM and related parameters.

5. To examine the effectiveness of dietary supplements of the sulfur amino acids L-methionine and L-cysteine in overcoming any indications of lipotrope inadequacy or methyl donor deficit in rats fed SBTI and the antilipotropic agent ethionine.

II. LITERATURE REVIEW

Legume seeds are the major and most economical source of protein for much of the world's population and serve as good sources of complex carbohydrates, minerals, dietary fibre and B vitamins (Protein Advisory Group of the United Nations 1973, Sgarbieri and Whitaker 1982, Gupta 1987). Dry beans are key components in the traditional diets of countries of Asia, Africa and Central and South America (Reddy et al. 1985) and the intake of legumes in Western countries is not only on the increase but also is recommended because of beneficial contributions to important indicators of nutritional status and to dietary control of diabetes (Anderson and Ward 1979, Erdman and Fordyce 1989).

The soybean (Glycine max (L.) Merrill) is the leading source of high quality plant protein and edible oil of the United States and several other countries and has great potential in meeting the protein needs of the world (Smith and Circle, 1972a). Isolated soy proteins and protein concentrates are established food ingredients that have widespread application for functional and nutritional purposes. In addition, the use of more traditional soy products such as tofu and tempeh by North American vegetarians and other health conscious individuals appears to be on the increase (Wang 1984).

Soy protein isolates have been shown to be completely adequate in meeting the protein maintenance requirements of adult humans (Scrimshaw et al. 1983). In addition, soy isolate-based textured foods and meat analogues are well accepted and generally retain the high nutritional quality of the isolates from which they derive. Soy isolate and soybean flour supplements are also widely used in formulation of comminuted meat products, and in breads, rice and vegetable protein mixtures. Functional attributes of soy preparations include

capacities as gelling, foaming and aerating agents (Smith and Circle 1972b, Wolf and Cowan 1975, Erdman and Fordyce 1989, Waggle et al. 1990).

Soy-based infant formulas are perhaps the best example of use of soy derivatives as a major dietary component. These may serve as a sole source of nutrition and can provide nutritional support for infants with temporary or congenital lactase deficiency or galactosemia, can serve as nutritional alternatives for infants of vegetarians and can be used prophylactically to prevent cow's milk allergy (Wink 1985).

The presence of a variety of naturally occurring toxic or antinutrient factors in soy and other legumes, can result in potentially adverse nutritional, biological and physiological responses in animals consuming them (Rackis 1972). Some factors occur only in individual species of plants, others may be in concentrations too low to have any nutritional significance, or may affect only genetically predisposed individuals (Sgarbieri and Whitaker 1982, Bender 1987, Gupta 1987, Liener 1990).

The levels of many antinutrients in food legumes can be reduced by suitable processing (Anderson et al. 1979, Gupta 1987). Because of the need to balance reduction of antinutrients with potential adverse changes to nutritional quality, however, residual levels often remain. When combined with increased use of legume products and the potential for underprocessing, significant intakes of plant-derived antinutrients can occur.

In soybeans, biologically active components having potentially deleterious effects in animals include protease inhibitors, hemagglutinins, allergenic factors, goitrogenic factors, flatus factors, estrogenic factors, and phytates (Rackis 1972, Anderson et al. 1979, Gupta 1987).

Protease or proteinase inhibitors and, in particular trypsin inhibitors, have long been considered the main antinutrient in soybeans (Rackis 1972). Low levels of several other

factors in soy products have not been shown to have major antinutritional significance for animals or to be readily controlled by suitable processing or supplementation.

Hemagglutinins (lectins) for example are glycoproteins which can bind to carbohydrate residues in cell membranes and have the capacity to agglutinate red blood cells. Ingestion of hemagglutinins of Phaseolus beans results in growth inhibition, reduced nitrogen digestibility and even death in several species (Liener 1974). Their antinutritional influence in raw or processed soy products, however, is less important. Rackis (1972) reported them to be more susceptible than trypsin inhibitors to gastric digestion and only minor changes in growth, Protein Efficiency Ratio (PER) and pancreatic hypertrophy occurred in rats fed raw soybean extracts from which hemagglutinins had been removed by affinity chromatography (Turner and Liener 1975, Liener 1976).

Tannins and associated polyphenols can inhibit several enzymes influencing digestion. Their contribution to trypsin inhibitor activity of soy products is also minimal. Tannin levels in soybeans have been reported to be negligible (Rao and Prabhavathi 1982, Sri Kantha et al. 1986) and Sessa and Bietz (1986) demonstrated that protein components of soybeans accounted for most of the trypsin inhibitor activity present. Suitable processing can generally eliminate tannin problems in legumes.

In vitro studies similarly, have demonstrated that presence or absence of phytates and phenolics (factors also capable of inhibiting protein digestibility) in soy protein isolates makes little difference on the in vitro digestibility of these products (Ritter et al. 1987).

Animal studies have shown that in addition to antinutrient effects, trypsin inhibitors present in raw soy and soy protein isolates can not only potentiate the effects of pancreatic carcinogens, but also by themselves initiate pancreatic cancer (McGuinness et al. 1980, 1981, Gumbmann et al. 1986). Because of the results of these animal studies and because

of increasing human intakes of legumes such as soy, the human health implications of dietary protease inhibitors remains an area of active study and concern (Roebuck 1987, Wormsley 1988).

Because of the potential for interaction or synergistic effects of these various factors both in terms of antinutrient and carcinogenic promotional effects (Chan and deLumen 1982b) it is most appropriate to study soy trypsin inhibitors as they occur in situ and not in isolated form.

While trypsin is an enzyme first isolated from the vertebrate pancreas, a number of trypsin-like enzymes having roughly similar functions exist widely in animals, plants and bacteria (Xavier-Filho and Campos 1989). In the literature, plant proteinase inhibitors having activity against trypsin and other animal proteases are often referred to interchangeably as protease inhibitors or trypsin inhibitors (Rackis 1972). The practise will be followed in this review.

Residual levels of protease inhibitor activity have been reported in a variety of processed legume products. Commercially available soy protein products for example, contain from 5 to 20% of the trypsin inhibitor activity of raw soybeans (Rackis and Gumbmann 1981). Combined with trypsin inhibitor activity present in other food products, human dietary intakes can be considerable. Doell et al. (1981) estimated that the average daily intake per person in the United Kingdom was 330 mg of trypsin inhibitor activity. In addition, certain groups such as infants fed soy-based infant formulas as a sole source of nutrition, vegetarians and individuals on legume-based, cholesterol-lowering diets may be exposed to relatively higher levels of these plant-derived antinutrients compared to the general population.

In order to appreciate the importance and potential for influence of protease inhibitors on animals, consideration must be given to their occurrence, functions, classification and to their effects on animals ingesting them.

1. PROTEASE INHIBITORS - OCCURRENCE

Naturally occurring protease inhibitors are ubiquitous and are found in plants, animals and microorganisms. In animals, endogenous protease inhibitors have been isolated or detected in pancreatic tissue (Green and Work 1953), in human lung tissue (Eriksson et al. 1965), and in testes and serum of vitamin E- and protein- deficient rats (Minakata et al. 1982).

These inhibitors have been isolated from the actinomycete fungus Neurospora crassa and are also found in a multitude of plant families and species, many having major significance as food and agricultural crops. Inhibitors are present in the pterydophyte Psilotum nudum and in higher forms such as members of the Gymnospermae, Gramineae, Solonaceae and the Leguminosae. Inhibitors occur in cabbage, cucumber, spinach and squash as well as in fruits such as tomato, pineapple, raisin, banana, plum and apple (Rackis et al. 1986, Xavier-Filho and Campos 1989).

From 5 to 10% of the soluble proteins in barley, wheat and rye grains are protease inhibitors (Mikola and Kirsi 1972, Ryan 1973) and some thirteen different protease inhibitors account for 15-25 % of the soluble protein of potatoes (Bender 1987). Sosulski et al. (1988) reported large species and cultivar differences in trypsin inhibitor activity in samples of wheat, oat, barley and rye with the higher activity in barley samples showing some resistance to heating.

Plant seeds, fruits, tubers, vegetative structures, roots and secretions have all been shown to contain protease inhibitors although legume seeds contain the highest levels (Green and Ryan 1972, Xavier-Filho and Campos 1989). Inhibitors comprise from 0.2-10% of total seed protein in edible dry beans of various species (Kakade 1974, Richardson 1977, Liener 1979, Sgarbieri and Whitaker 1982).

The widespread occurrence and variation in levels of protease inhibitors even within legume species and cultivars has been amply demonstrated. Trypsin inhibitors have been found not only in soybeans, but also in Vicia faba (faba beans) (Wilson et al. 1972), and species and varieties of Phaseolus including P. lunatus (lima beans) (Haynes and Feeney 1967), and P. vulgaris (kidney beans and navy beans) (Palmer et al. 1973, Wang and Chang 1988). Vigna species such as V. unguiculata (cowpea) (Ogum et al. 1980), V. mungo (black gram) (Kamalakannan et al. 1981), and V. sinensis (Valdebouze and coworkers 1980), as well Psophocarpus tetragonolobus (winged bean) (Kadam and Smithard 1987), and Cicer arietinum (chick pea) (Sastry and Murray 1987) also contain such inhibitors. All these legumes have potential or actual use as protein sources for man and other animals.

The up to five-fold variation in trypsin inhibitor levels or activities in varieties and strains of soybeans (Kakade et al. 1972, Gandhi et al. 1984) and faba bean cultivars (Marquardt et al. 1975) suggests the potential for control of levels by varietal selection. In some cases, the high trypsin inhibitor content of certain legumes may contribute to their high nutritional quality for particular animals because of high levels of essential amino acids and the specificity of their inhibition for only some proteinases of the species (Sastry and Murray 1987).

Concentration of inhibitors within plants varies not only with species and variety but also with age, physiological state and extent of insect damage. Seed development and

germination of cultivars of black gram (Urd bean) of India resulted in changes in trypsin inhibitor activity (Chitra and Sadasivan 1986) and Wilson and coworkers have demonstrated both an ordered series of proteolytic cleavages occurring in the trypsin inhibitors of germinating mung bean (Vigna radiata) seeds and a sequence of proteolysis and eventual disappearance of Kunitz isoinhibitors in soybean cotyledons during imbibition, germination and seedling growth (Lorensen et al. 1981, Wilson et al. 1988).

2. PROTEASE INHIBITORS - FUNCTIONS

The ubiquitous occurrence of protease inhibitors in nature emphasizes the fundamental importance of proteolysis in biological systems and the need to control such proteolysis. As described most succinctly by Laskowski (1986) 'We wish to eat the other guy's pancreas or intestine-not our own...we wish our blood to clot at specific locations but not in the entire circulatory system....'. Roughly similar requirements can be inferred in plants.

In animals, inhibitors likely protect against inappropriate proteolysis. Pancreatic protease inhibitors may prevent pancreatic self digestion by activated zymogens (Green and Work 1953) just as α1-proteinase inhibitor of human lungs prevents excess connective tissue turnover and emphysema via inactivation of neutrophil elastase (Eriksson 1965). Cysteine protease inhibitors of several animal species appear to control proteinases involved in inflammatory response and myofibrillar protein degradation (Swaim and Pizzo 1988) and increase in α1-cysteine protease inhibitors in serum and testes of vitamin E and protein deficient rats (Minakata et al. 1982, Minikata et al. 1987) may reflect a homeostatic response to lysosomal membrane breakdown and increased protein degradation.

Plants must also control their own proteolysis and perhaps discourage proteolysis of themselves in the digestive systems of potential consumers. Control of proteolysis is required for protein synthesis and turnover, for protein destination (removal of signal peptide) and to facilitate various transport mechanisms.

Ashton (1976) suggested that protease inhibitors may be one of several factors controlling plant protein mobilization. Changes in trypsin inhibitor levels or activities during seed development, germination and early growth of legumes such as black gram, soybeans and varieties of Phaseolus vulgaris (Collins and Sanders 1976, El-Hag et al. 1978, Chitra and Sadasivan 1986, Nielsen and Liener 1988) suggest roles in maintenance of seed dormancy, in control of seed development and in ordered proteolysis of storage proteins to amino acids and derivatives for energy and synthetic requirements of the developing plant.

Inhibitors themselves may serve as plant storage reserves of protein and amino acids. In particular, they may be major plant stores of sulfur amino acids since 30 to 40% of total lima and navy bean cystine is found in trypsin inhibitors (Kakade at al. 1969) and up to 19% of amino acid residues of Bowmnan-Birk isoinhibitors in soybeans are cysteine (Hwang et al. 1977).

Initial changes in levels of specific Kunitz and Bowman-Birk inhibitor isoforms during germination and seedling growth involves limited specific hydrolysis (Madden et al. 1985, Hartl et al. 1986). In the case of Kunitz inhibitors of soy these changes were suggested as too slow to indicate a storage source of amino acids (Freed and Ryan 1978). Longer term studies, however, demonstrated that inhibitors of both types increase with maturation of legume seeds and undergo gradual or precipitous decrease during germination (Pusztai 1972, Wilson et al. 1988) indicating that both classes could potentially serve as energy or protein synthetic sources.

Inhibitors may also act as defense mechanisms against the proteases of microbes, insects or animal herbivores thereby enhancing survival the plant or of the plant species. Wilson (1980) suggested that the release of trypsin and chymotrypsin inhibitory activity during the germination of the seeds of several legumes might not only inhibit pathogenic microorganisms but also stimulate the establishment of beneficial symbionts in legume rhizospheres. The rapid accumulation of protease inhibitors in leaves of potatoes and tomatoes subjected to mechanical or insect damage (Green and Ryan 1972) and reduced growth performance of insects such as the corn earworm and beet armyworm exposed to low concentrations of protease inhibitors (Broadway et al. 1986) support a defensive role for inhibitors. Insecticidal capacity of inhibitors at high concentrations has also been reported (Hilder et al. 1987). Janzen et al. (1976, 1986) suggested that the build up of inhibitors along with other constitutive chemicals in seeds and other plant components, could discourage further insect infestation or attack.

Evolution of and selection for the presence of protease inhibitors may have in part resulted from their capacity to deter ingestion of the legume or to enhance the undigested passage of legume seeds through the guts of herbivores thereby enhancing dispersal of the plant species and contributing to its potential survival (Green and Ryan 1972, Ryan 1973, Birk 1985, Rothman 1986, Janzen et al. 1986).

Synthesis or occurrence of protease inhibitors in different plant parts can be subject to endogenous developmental control or to external environmental influences. This control may have a multifunctional role for plant protease inhibitors. Certain inhibitors could prevent activity or utilization of endogenous plant proteins in the dormant seed, while at the same time serving as storage reserves for amino acids. The capacity of inhibitors to inactivate animal and insect proteases could also lessen insect attack or prevent successful digestion

of the seeds by herbivores. Synthesis or release of other inhibitors at sites of insect or mechanical damage could also prevent further microbial or insect damage. The economy of such a multifunctional role for inhibitors occurring in plants would have obvious advantages and would likely be selected and genetically preserved.

Wilson et al. (1988) described changes during germination, in levels of proteases specific for initial proteolysis of Kunitz inhibitor proteins in soybean cotyledons. Their action could change inhibitor conformation resulting in change in activity and exposure to further enzymatic degradation. Their degradation could result in release of enzyme-inhibitor complexes, enzyme activation or proteolysis of other storage proteins in the developing plant.

3. PLANT PROTEASE INHIBITORS - CLASSIFICATION

In plants, protease inhibitors have thus far been detected in four specific mechanistic classes (corresponding to the proteases they inhibit). These include: serine protease inhibitors; sulfhydryl (cysteine) protease inhibitors; aspartic acid protease inhibitors and metalloprotease inhibitors. The serine protease inhibitors are the best known and have been isolated and characterized from several species (Laskowski and Kato 1980, Laskowski 1986).

a) Serine Protease Inhibitors

The inhibitors of this class have been further subdivided into a number of families partly based upon source and homologous features involving position of disulfide bridges and reactive sites. Plant serine protease inhibitors are grouped in at least six families: the Kunitz-type trypsin inhibitors and the Bowman-Birk protease inhibitors of soybean (and other legumes); the I and II inhibitor families of potato (Laskowski and Kato 1980); the squash inhibitor family (Wieczorek et al. 1985) and the α-amylase/trypsin inhibitor family (Odani et al. 1983). Laskowski (1986) has conservatively classified five families of serine protease inhibitors found in animals and two families from microorganisms.

The Kunitz and Bowman-Birk families of serine protease inhibitors are among the most studied and best characterized of the inhibitors perhaps reflecting their presence in important food legumes and their being among the first to be discovered and isolated. The members of these two families have been separated into several classes and several isoinhibitor forms of each have also been reported (Tan-Wilson and Wilson 1986, Tan-Wilson et al. 1987). The

serine denoting this class of inhibitor refers to the activated residue on the protease molecule acting as the catalytic site during proteolysis.

Because of the increasingly important role of legumes and in particular soy-based products in human diets, the study of protease inhibitors such as trypsin inhibitors of soybean is of ongoing interest. Nevertheless it must be emphasized that appreciable levels of protease inhibitors are contributed by other food components of the diet such as potatoes and even cooked eggs (Doell et al. 1981, Rackis et al. 1986).

b) Legume Families of Serine Protease Inhibitors

Members of two families of serine protease inhibitors are often described by the general term of soybean trypsin inhibitor (SBTI) or trypsin inhibitor, perhaps based on the first animal protease against which they were tested. They are more properly separated into Kunitz-type and Bowman-Birk-type forms based upon molecular weight, amino acid composition, enzyme specificity and heat stability. As for other serine protease inhibitors they act as very specific substrates, competitively forming relatively stable 1:1 complexes with the enzymes they inhibit by means of a peptide bond (reactive site peptide bond) at each inhibitory domain of the inhibitor molecule. One or more specific enzymes may compete for or be inhibited by the same site or by different sites on an inhibitor molecule (Laskowski and Kato 1980).

From 10-15 amino acid residues of the inhibitor protein may be in contact with the active region of susceptible proteinase and this affects both the specificity and strength of the enzyme-inhibitor complex (Laskowski 1986). Rackis (1972) reviewed the initial interaction of Kunitz inhibitor of soy with trypsin. Cleavage of the Arg(64)-Ile(65) peptide bond in the

inhibitor polypeptide chain by trypsin enzyme is involved. The presence of an arginine or lysine residue in the active site of the inhibitor molecule appears to be required for activity and adjacent tyrosine or tryptophan residues also have an influence.

i) Kunitz Family of Inhibitors

Kunitz (1947) described the properties of the protein which defines this class of trypsin inhibitor. The Kunitz inhibitor (KTI) of soybean is a globulin protein with an isoelectric point of 4.5-4.6 and a molecular weight of about 21,500 daltons. It consists of a single polypeptide chain of 181 amino acids including two methionine and four cysteine residues. The cysteine residues contribute two disulfide bonds and more glycine, valine, leucine, isoleucine and arginine residues are present than found in Bowman-Birk inhibitor (Sgarbieri and Whitaker 1982, Xavier-Filho and Campos 1989).

Three genetic variants of soy KTI having different amino acid residues at particular sites on the polypeptide chain have been described (Kim et al. 1985). Tan-Wilson and Wilson (1986) indicate that these variants differ somewhat in total numbers of amino acid residues which may partially explain reported variations in molecular weights.

Kunitz inhibitors are mainly specific for interaction with trypsins of various animal species. They do not react with carboxypeptidases, thiol proteases, pepsin, thrombin or collagenase. Association equilibrium constants for the three Kunitz inhibitor soy variants range 1000-fold in terms of complex formation with bovine trypsin (Freed and Ryan 1980) and likely also in terms of complex formation with trypsins of other animal species. The isoinhibitor forms are present in different amounts in different soybean cultivars (a limit of two forms per cultivar) and germination results in slight changes in composition because of

proteolytic digestion of carboxyl-terminal amino acid residues of the inhibitor molecules (Tan-Wilson and Wilson 1986). One soy cultivar has been developed which is completely lacking in Kunitz inhibitor although it retains both trypsin and chymotrypsin inhibiting capacity due to presence of Bowman-Birk inhibitors (Hymnowitz 1986).

Several other Kunitz-type inhibitors with similar molecular weights (about 20,000 daltons) have been characterized from the seeds of other legumes. Many of them also exist as isoinhibitors with high degrees of sequence homology (Barnes 1987, Xavier-Filho and Campos 1989).

Kunitz inhibitors have been considered the most susceptible of soy inhibitors to inactivation by heat processing or by the acidic conditions of the vertebrate stomach (Sgarbieri and Whitaker 1982, Birk 1985, Tan-Wilson and Wilson 1986). This opinion was based on findings of Rackis (1966) using ion exchange-purified inhibitor and those of Obara and Watanabe (1971) using low temperature (36 to 70 ° C) heating of aqueous solutions of purified inhibitors. The relatively low cysteine content resulting in fewer disulfide bridges and a less compact molecule compared to Bowman-Birk inhibitor (BBI) could contribute to such lability.

More recent evidence using enzymatic and immunochemical techniques confirms that purified KTI are more heat labile (when heated in aqueous solutions at 100 ° C) than are purified BBI inhibitors. KTI present in soy flour and equilibrated to water activities (a_w) of 0.50 and 0.75, were found, however, to be much more heat stable than were BBI also present. Moreover, purified BBI added to soy flour at 0.75 a_w and heated, was more rapidly inactivated than was added KTI (DiPietro and Liener 1989). Sessa and Bietz (1986) using a variety of isolation techniques on toasted soybean flour extracts, found that proteinaceous material accounted for the majority of the residual trypsin inhibitor activity and that both Kunitz and

Bowman-Birk inhibitors were present. Brandon and colleagues (1988) using an enzyme-linked immunoassay (ELISA), also demonstrated the occurrence of residual Kunitz inhibitors in processed soy products including concentrated infant formulas. Thus residual activity in heat treated soy or other legume products can involve both Kunitz and Bowman-Birk inhibitors, although Friedman et al. (1989) using the results of his studies with Brandon et al. (1988) suggests that Kunitz inhibitors contribute less than 10% of residual trypsin inhibitor activity in soy infant formulas.

Chemical changes resulting from autoclaving of soy flour and microenvironmental influences such as location, potential interactions with free thiol groups and other proteins or carbohydrates, operating on in situ inhibitors may account for the stability differences noted above (Friedman et al. 1984, DiPietro and Liener 1989).

ii) Bowman-Birk Family of Inhibitors

The existence of a second antitryptic factor in soybeans was reported by Bowman (1946). Birk (1985) reviewed some 40 years of research involved in the isolation and characterization of the soybean Bowman-Birk inhibitor (BBI) which represents this family of plant serine protease inhibitors. It consists of a polypeptide chain having a molecular weight of 8000 daltons and 71 amino acids. BBI-type inhibitors in other legumes range in molecular weight from 6000 to 10,000 daltons although because of a tendency for molecules to self associate, higher molecular weights have been reported. The presence of 14 cysteine residues involved in 7 disulfide cross links gives the BBI molecule a very compact structure as well as making it a relatively rich source of dietary sulfur amino acids compared to other

legume storage proteins. The BBI of soy was determined to have an isoelectric point of 4.2 which is slightly lower than that of KTI (Birk et al. 1963).

As for other members of this family, the soy BBI is a double-headed inhibitor having two independent enzyme inhibition sites-one against trypsin and one against chymotrypsin. Inhibitory activity against elastase has also been reported. Sequence homology around the inhibitory sites suggests gene duplication from a single-headed ancestral inhibitor. Sequence homology also occurs between inhibitors of different legume species (Rackis et al. 1986). A 1:1 complex is formed with trypsin or chymotrypsin or a ternary complex when both enzymes are bound. A soybean BBI inhibitor that inhibits two moles of trypsin per mole of inhibitor and does not inhibit chymotrypsin has also been isolated (Odani and Ikenaka 1976).

Several BBI isoinhibitor forms exist with individual soybean cultivars having from five to twelve different isoinhibitors (Hwang et al. 1977, Tan-Wilson and Wilson 1986). These isoinhibitors have subsequently been classed into four different subgroups based upon isoelectric points, amino acid compositions, molecular weights, relative abilities to inhibit bovine trypsin, chymotrypsin and elastase and immunological cross-reactivity (Tan-Wilson et al. 1987).

As previously mentioned, BBI were considered to account for residual trypsin and chymotrypsin activity in heated soy samples and to be more resistant to acid conditions of the vertebrate stomach. More recent work, however, suggests that both KTI and BBI can contribute to residual protease inhibitor activity in soy-derived food products (DiPietro and Liener 1989, Brandon et al. 1988, 1989).

Bowman-Birk type inhibitors have been isolated from several other legumes. Black-eyed peas possess two different isoinhibitors of Bowman-Birk size and characteristics (Gennis and Cantor 1976), each having two inhibitor sites although one has both sites specific for

trypsin and the other, separate chymotrypsin and trypsin binding sites. Kortt (1979) isolated three proteins having trypsin inhibitor activity, high cysteine levels and molecular weights in the BBI range from seeds of winged bean (Psophocarpus tetragonolobus). The main trypsin inhibitors of chick pea isolated by trypsin-Sepharose 6B affinity chromatography were also low molecular weight (8000-9000 daltons), double headed BBI forms. In the latter isolate, both binding sites were found to be specific for trypsin and it was suggested that the essential amino acid content of these inhibitors and their inability to bind chymotrypsin contributed to the high nutritional quality of this variety of chick peas (Sastry and Murray 1987). Weder and Mueller (1989) described two Bowman-Birk type isoinhibitors in lentils, each with a single independent reaction site for trypsin and for chymotrypsin. Both isoinhibitors lacked methionine and tryptophan and were high in cystine content with seven to eight disulfide bridges.

As will later be described, the relative levels of Bowman-Birk and Kunitz inhibitors as well as the relative levels of individual isoinhibitors in a particular legume can be of considerable nutritional importance to animals consuming the legume.

4. EFFECTS OF PLANT PROTEASE INHIBITORS IN ANIMALS

a) Short-term Antinutritional And Physiological Effects

Historically, the area of most interest in the study of protease inhibitors has been their antinutritional effects on animals. Osborne and Mendel (1917) first observed that feeding raw or inadequately cooked soybeans would not support the growth of rats. The isolation of a heat-labile inhibitor of trypsin in soybeans that when fed to rats also decreased growth and the demonstration that the nutritive value of soybean flour heated at different temperatures, improved in proportion to the destruction of this factor pointed to trypsin inhibitors as the main cause of the growth failure (Kunitz 1947, Borchers et al. 1948, Westfall and Hague 1948, Rackis 1965, Rackis et al. 1975).

Booth et al. (1960) reported that short term intakes by rats of raw soybean meal-based diets resulted not only in poor growth and decreased feed efficiency but also in pancreatic hypertrophy relative to rats fed casein or heated soybean meal-based diets. Increased pancreatic protein synthesis and secretion of enzymes in rats because of dietary intakes of raw soy or purified soybean trypsin inhibitors or with gastric infusion of trypsin inhibitors has also been demonstrated (Barnes and Kwong 1965, Rackis 1965, Green et al. 1973, Schneeman et al. 1977) and numerous researchers have confirmed that trypsin inhibitors present in soybeans and other legumes are responsible for a variety of interrelated effects in susceptible animals including reduced growth, decreased protein digestibility, inhibited proteolysis, depressed metabolizable energy, reduced fat absorption, reduced availability of minerals, vitamins and amino acids, enhanced pancreatic synthesis of proteins, phospholipids and nucleic acids, hypersecretion of pancreatic enzymes, and pancreatic hypertrophy and

hyperplasia (Rackis 1974, Nitsan and Liener 1976a, 1976b, Abbey et al. 1979a, 1979b, Rackis and Gumbmann 1981).

i) Species Differences

Animal species vary in their susceptibility to the adverse nutritional and physiological effects of ingestion of trypsin inhibitors. Some animals appear extremely sensitive (Nitsan and Nir 1977, 1986) and this has can have practical and economic implications in animal production in terms of the use of a particular legume.

The response of chickens to ingestion of raw soy or isolated trypsin inhibitors is similar to that of rats and because soybean meal is the major protein supplement of practical poultry rations (McNaughton et al. 1981), the nutritional implications of active trypsin inhibitors for poultry are important. Chernick et al. (1948) reported reduced growth, pancreatic enlargement and increased stores and secretion of pancreatic enzymes in chicks fed raw soybean meal containing trypsin inhibitors. Diets containing purified soy trypsin inhibitors, soybean whey proteins, defatted soybean meal fractions or strains of soybeans high in trypsin inhibitors have subsequently also been shown to cause increases in pancreatic weight and secretion and in stimulation of gall bladder contraction of chicks. Methionine supplementation of raw soy meal was found to improve growth performance but resulted in even greater pancreatic hypertrophy than in chicks fed raw soy without added methionine (Niess et al. 1972, Bajjalieh et al. 1980). Trypsin inhibitor-containing field beans (Vicia faba) have a similar trophic effect on the chick pancreas (McNab 1977).

Chemical (DNA, RNA and protein) and histological analysis indicated that the pancreatic enlargement occurring in chicks with short term (up to 6 weeks) feeding of raw soybean meal

resulted from hyperplasia (increased pancreatic acinar cell numbers) (Kakade et al. 1967, Salman et al. 1968) and hypertrophy (increased acinar cell diameter) (Saxena et al. 1963).

A comparative study of goslings and chicks fed semisynthetic diets based on raw or heated soybeans showed that goslings are more sensitive to raw soybeans. Greater depression in weight gain and increased organ weights as well as pancreatic hypertrophy and hypersecretion occurred in goslings. Inadequate feathering, not seen in chicks, also occurred (Nitsan and Nir 1977).

Both chickens and rats appear to adapt to continued intakes of raw soy flour since growth rate and food conversion efficiency in both species improve over time (Bornstein and Lipstein 1963, Nitsan and Liener 1976a).

Mice are also susceptible to short term intakes of soy trypsin inhibitor. Weight gain, Protein Efficiency Ratio, and intestinal trypsin activity decreased and pancreatic weight, pancreatic trypsin, chymotrypsin and amylase increased after 1 month in weanling mice fed casein-based diets with added soy- or potato-derived trypsin inhibitors (Roy and Schneeman 1981, Gumbmann et al. 1989). Growth inhibition occurring in mice fed increasing amounts of soy trypsin inhibitor combined with low dietary nitrogen was prevented by increased dietary nitrogen provided by intact casein or equivalent amounts of free amino acids (Gumbmann et al. 1989).

Golden Syrian hamsters fed raw soy flour-based diets compared to those fed heated soy flour low in trypsin inhibitors, similarly had depressed growth, decreased feed efficiency, decreased apparent protein digestibility, increased relative pancreatic weight and increased content and activity of pancreatic-derived proteases in pancreas, small intestine, cecum, large intestine and feces (Hasdai and Liener 1983).

In young guinea-pigs fed trypsin inhibitors, growth depression occurred without any pancreatic enlargement. Hasdai et al. (1989) found reductions in feed consumption, feed conversion efficiency, and body weights without increase in pancreatic weight, in young guinea-pigs fed raw soy flour-based diets containing active trypsin inhibitors compared to those fed cooked soy flour low in trypsin inhibitors. Lower pancreatic and intestinal levels of proteinases and amylases in the raw soy-fed animals suggested hyposynthesis and/or hyposecretion of pancreatic enzymes.

Swine do not appear to undergo pancreatic enlargement or increased synthesis of pancreatic enzymes in response to dietary trypsin inhibitors. Gilts fed raw soybean meal high in trypsin inhibitors or heated soybean meal with added Kunitz inhibitor had reduced growth but no pancreatic enlargement compared to animals fed heated soybean meal. Decreased pancreatic and intestinal trypsin and chymotrypsin activities also occurred. Greater enzyme inhibition, however, in pigs fed raw soybean compared to those fed heated soybean meal with added inhibitor indicated the influence of other soy components on the inhibition (Yen et al. 1977).

Weanling female swine fed raw soy flour diets, also had lower growth but no pancreatic enlargement, decreased nitrogen digestibility and increased fecal trypsin compared to swine fed casein, heated soy flour or soy protein isolates much lower in trypsin inhibitor contents. Pancreatic composition of the pigs fed raw soy did change, with increased RNA, DNA and decreased protein, and ratios of protein/DNA, protein/RNA and RNA/DNA. Lower pancreatic trypsin and chymotrypsin levels were also measured. Pair-feeding trials using casein and raw soy flour indicated that reduced food intake had only slight influence of the observed differences (Struthers et al. 1983a).

A negative feedback inhibition mechanism on pancreatic enzyme secretion (a key feature of susceptible species' pancreatic response to trypsin inhibitors) has been reported in the pig (Corring 1974) although unlike the rat, the pig does not appear sensitive to injections of cholecystokinin (Struthers et al. 1983a). Cholecystokinin (CCK) is the proposed intermediary of feedback inhibition and pancreatic trophic response to trypsin inhibitors.

Cows are also influenced negatively by ingestion of raw soybeans. Young Holstein bull calves exhibited depressed growth, reduced feed efficiency and decreased protein and fat digestibility when fed raw versus heated soybeans. However, pancreatic weights, trypsin and chymotrypsin activities were not affected by feeding raw soy, heated soy or heated soy with added trypsin inhibitor which contrasts to findings in rats and chicks (Kakade et al. 1974). Sissons (1982) found that crudely processed soybeans were unsuitable as a protein source for calves, causing gastrointestinal disturbance, growth deficit and death. Immunological hypersensitivity reactions caused by major globulin proteins (glycinin and β-conglycinin) present in soy were proposed as a likely cause.

Dogs, like growing pigs and unlike chicks and rats, appear to be resistant to the pancreatic hypertrophic effects of soy trypsin inhibitors. Feedback inhibition of pancreatic proteases on pancreatic enzyme secretion was not seen in chronic and acute studies of dogs. In contrast to trypsin inhibitor-sensitive species, increased pancreatic secretory response with intravenous infusion of exogenous hormones such as secretin and CCK did not occur in dogs chronically fed trypsin inhibitor-containing diets (Sale et al. 1977). Dietary amino acids appear to be more potent stimulators of pancreatic secretion (Meyer et al. 1976), perhaps reflecting higher levels of free amino acids in carnivore food sources compared to storage proteins consumed by other animals.

No significant change in pancreatic weight or cellular structure occurred in adult dogs fed 15 or 30% raw soybean meal over 8 weeks compared to dogs fed a control diet. A temporary decrease in pancreatic enzyme synthesis seen after 8 weeks in dogs fed 15% raw soy-based diet did not occur at 12 weeks and was not seen in dogs fed the 30% raw soybean diet. It was suggested that the higher protein of the 30% raw soybean diet overcame dietary amino acids deficits and that the dog could also adapt to such dietary deficits over time by mobilization of amino acids from body stores (Patten et al. 1971). Whether the adaptive capacity of adult dogs to chronic intakes of soy trypsin inhibitors occurs in younger animals remains to be demonstrated.

Feeding raw soy compared to heated soy flour or casein had least physical and biochemical effects in female rhesus (Macaca mulatta) monkeys compared to weanling rats and pigs. No growth depression, increase in pancreatic size, protein, RNA or DNA content or histological changes were observed. As for weanling pigs, fecal trypsin levels decreased in response to raw soy flour (Struthers et al. 1983).

Ausman et al. (1986) fed cebus (Cebus albifrons) monkeys diets based on low trypsin inhibitor soy protein isolate for up to 4 years. Compared to monkeys fed lactalbumin- or casein-based control diets, these animals showed no differences in growth, clinical chemical, hematological or pancreatic histological and biochemical parameters.

The latter authors suggested that primates may be less susceptible than rats to the adverse effects of chronic feeding of trypsin inhibitors. The trypsin inhibitor content of the soy isolate diet used in their study, however, was less than that found in soy protein isolate diets used in chronic USDA studies of rats (Rackis et al. 1985). In addition, the monkeys used in both studies were above weanling age and it is questionable if results obtained should

be compared to those obtained with weanling pigs and rats or be extrapolated to infant monkeys or other infant primates.

The physiological response of humans to ingestion of high levels of trypsin inhibitors either in pure form or present in processed or raw legume products, has not been extensively studied and in the case of human infants, appears entirely lacking. The existence, however, of feedback inhibition on human pancreatic protease output and the influence of soy trypsin inhibitors on this output has been established.

A cancer patient with normal pancreatic output blocked and bearing a bile-duct catheter, was found to have trypsin-induced feedback inhibition of pancreatic secretion suppressed by intraduodenal infusion of soybean trypsin inhibitor in combination with bile-pancreatic juice (Ihse et al. 1977). Similarly, acute studies of normal adult males and females showed that pancreatic output and activities of trypsin, chymotrypsin, elastase and amylase could be significantly increased by duodenal infusion of soy BBI or by infusion of pure human pancreatic juice in which trypsin and chymotrypsin activities had been abolished by BBI (Goodale et al. 1985, Liener et al. 1988).

Marked increases in plasma CCK levels in eleven healthy subjects fed meals containing raw soy flour high in trypsin inhibitor compared to the same subjects fed heated flour with lower inhibitor levels were noted by Calam et al. (1987). The release of CCK into the blood from intestinal mucosa cells in response to low levels of free trypsin is a key feature of the feedback mechanism controlling pancreatic enzyme synthesis and release in species susceptible to the effects of trypsin inhibitors.

Several factors may be involved in the variation in responses of animals to dietary trypsin inhibitors. Differences in control mechanisms for synthesis of pancreatic nucleic acids and enzymes in different species were suggested by Struthers et al. (1983a). Based upon a

review of the literature, it has been proposed that species' susceptibility to pancreatic trophic response was related to relative pancreatic weight, enlargement occurring only in species with pancreatic weights greater than 0.3% of body weight. Rats, chicks, mice and immature guinea-pigs with relative pancreatic weights ranging from 0.29 to 0.8% were reported to be susceptible, whereas calves, pigs and dogs with relative pancreatic weights from 0.06 to 0.24 were not. The human pancreas (0.09 to 0.12% of body weight depending upon age, according to this hypothesis, should thus not undergo hypertrophy with ingestion of dietary trypsin inhibitors (Kakade et al. 1976, Liener 1979). Human empirical studies are not available, however, and there are indications that frequent meal feeding, as occurs in the human infant, may increase the effects of trypsin inhibitors in animals (Nitsan and Nir 1986).

There are both species and age differences in relative requirements for essential amino acids and in pancreatic response to ingestion of soybean trypsin inhibitors. The lower growth, poor feather development and greater pancreatic enlargement in goslings compared to chicks fed raw soy with high contents of trypsin inhibitors, was taken to reflect the higher sulfur amino acid requirements of the goslings (Nitsan and Nir 1977). Higher sulfur amino acid requirements for growth in rats compared to humans, and in human infants compared to adults (Bodwell 1978, FAO/WHO/UNU 1985, FAO/WHO 1990) similarly may exacerbate the negative effects of decreased protein digestibility and amino acid availability due to trypsin inhibitors. The greater pancreatic trophic response of young versus older rats and the very rapid induction of pancreatic hyperplasia and hypertrophy occurring in suckling rats due to trypsin inhibitor feeding (Rackis 1965, Melmed et al. 1976) as well as the improved growth and feed conversion efficiencies in rats and chicks with continued dietary intakes of inhibitors (Bornstein and Lipstein 1963, Nitsan and Alumot 1964, Nitsan and Liener 1976), may reflect decreasing sulfur amino acid requirements of older animals.

The susceptibility to soybean protease inhibitors or to protease inhibitors in general may also be a function of the relative importance of different proteinases in the digestive processes of a species. Considerable variation existed in the activation, activity and pattern of trypsin, chymotrypsin and total proteases in pancreatic extracts of different animal species. The relative contributions of trypsins + chymotrypsin, elastases and carboxypeptidases were 50, 25 and 25% respectively, in human pancreatic extracts, whereas in bovine extracts the same components accounted for 75, 20 and 5% respectively, of total proteolytic activity (Prabhu et al. 1984). In fox pancreatic extracts, trypsin alone accounted for 67% of total proteolytic activity (Krogdahl and Holm 1983).

Diet differences may have influenced these findings since it has been postulated that differential production and secretion of pancreatic enzymes occurs subject to dietary protein quality and amino acid pattern (Adelson and Rothman 1974). Weder (1986) has also cautioned that both the substrate selected and the ratio of inhibitor to enzyme used in in vitro assays of proteinase inhibitor activity can influence results.

Bovine trypsin is used in standard assays for determination of trypsin inhibitor levels or activities in soy and other legumes (Kakade et al. 1974, Smith et al. 1980, Hammerstrand et al. 1981). The extent of inhibition of bovine trypsin by an inhibitor from a particular legume may, however, not reflect its potential influence on digestion and related physiological events in humans and other species.

In vitro studies reveal a species variation in susceptibility of individual proteases to inhibition by proteinase inhibitors. Feeney et al. (1969) found varying inhibition of both human and bovine trypsins by a variety of naturally occurring inhibitors of both plant and animal origin, with weak inhibitors of human trypsin frequently being strong inhibitors of bovine trypsin. Differential inhibition of proteases of monkey, rat, cow and man by soybean

trypsin inhibitors were reported by Struthers and MacDonald (1983) and Weder and Meuller (1989) found human chymotrypsin to be more strongly inhibited than bovine chymotrypsin by the two major Bowman-Birk-type inhibitors isolated from lentils.

Individual isoforms of proteases within an animal species also differ in susceptibility to inhibition by the protease inhibitors. Only one of two isolated anionic forms of human trypsin were completely inhibited by soybean KTI (Figarella et al. 1975). Homogenous preparations of the more important cationic form of human trypsin showed strong inhibition by lima bean trypsin inhibitor (a Bowman-Birk inhibitor) and by KTI but only weak inhibition by soybean BBI (Mallory and Travis 1975).

Crude pancreatic extracts (considered to more closely resemble the in vivo situation compared to purified enzyme preparations) similarly show differences in inhibition of component proteases depending upon the animal source of the pancreatic extract and upon the source of legume protease inhibitor extract added. Of ten different potential food legumes, extracts of soy, field bean, kidney bean and bengal gram were more active against bovine than human trypsin and chymotrypsin whereas those of cowpea and redgram were more effective at inhibiting human chymotrypsin (Prabhu et al. 1984). In a more extensive study using pancreatic preparations from 10 different mammalian species, Bhat and Pattabiraman (1986) found wide variation in susceptibility of total proteolytic, trypsinolytic and chymotrypsinolytic activity to inhibition by extracts from seeds of different species of Indian legumes. Krogdahl and Holm (1983) also determined that the sensitivity of total proteolytic activity of pancreatic enzyme extracts from different animal species to Kunitz soybean inhibitor varied ten fold. The human preparation was found to be the least sensitive of the various animal preparations tested. Animal species may thus differ in their relative tolerance to levels of particular inhibitors present in different legume food sources.

Finally, the relative levels of particular inhibitors in different legumes and their relative resistance to processing or digestion may be key determinants of their antinutritional influence in animals. As previously described, legume species and strains vary in total trypsin inhibitor activity as well as in contents of different classes and isoforms of inhibitors (Kakade et al. 1972, Odani and Ikenaka 1976, Tan Wilson and Wilson 1986, Hymnowitz 1986). The various classes and isoforms can differ not only in their affinity for the proteases of different animal species but also in their susceptibility to inactivation by processing and digestion (Yavelow et al. 1983, Weder 1986). These differences could result in strong or weak inhibition of digestive proteases and subsequent pancreatic and growth responses to ingestion of both raw and processed legumes by particular animals.

These findings have obvious implications in terms of the need for caution in extrapolating the effects of dietary protease inhibitors on one animal species to another and in terms of selection of legumes and the processing required for their optimal and safe use as foods for particular species.

The molecular basis for differences in susceptibility of species' proteases to inhibition by particular inhibitors likely reflects differential affinity and stability of enzyme-inhibitor complexes. Preciseness of fit of the inhibitor reactive sites with the enzyme active region may be involved. Stronger binding of human compared to bovine chymotrypsin at the chymotrypsin reactive site of the two major Bowman-Birk-type isoinhibitors of lentils and overlapping binding of human chymotrypsin at the trypsin reactive site resulted in greater inhibition of human versus bovine chymotrypsin. Similar overlapping binding by bovine trypsin at both sites also resulted in greater inhibition of bovine compared to human trypsin (Weder and Meuller 1989).

Estimates as to the relative contribution of protease inhibitors to the deleterious nutritional and pancreatic changes resulting from dietary intakes of raw or underprocessed soybeans vary. Grant et al. (1986) demonstrated that the whey fraction of raw soybeans containing the bulk of trypsin inhibitor and hemagglutinin activity, caused lowest net protein utilization of all soy fractions fed to rats. Feeding of raw soy extracts from which hemagglutins had been selectively removed, had earlier been reported to have minimal effects on rat growth performance (Turner and Liener 1975).

Kakade et al. (1973) using results from rats fed unheated crude soy flour extracts from which trypsin inhibitors had been selectively removed by affinity chromatography, determined that trypsin inhibitors accounted for only 40% of the growth inhibition and 40% of the pancreatic enlargement occurring. In vitro digestion studies by the same authors indicated that trypsin inhibitors also caused only 40% of the protein resistance to trypsin present in the raw extract. The balance of the negative effect was reported to result from resistance of soy proteins to the digestive enzymes of the rat and perhaps to the presence of other inhibitors.

Rackis et al. (1975) estimated that Kunitz soy trypsin inhibitor caused 30-50% of the growth inhibitory response and most of the pancreatic hypertrophy occurring in rats fed raw soybeans. Temler et al. (1984a) using rats fed casein diets with added Kunitz inhibitor equivalent to those in raw soy, similarly reported pancreatic enlargement but only slight indications of other antinutritional effects. The high protein level and quality of the diet used by the latter authors, however, likely compensated for many of the antinutritional effects of trypsin inhibitors and prevented potential synergistic influences with other components of soy.

In contrast, Saxena et al. (1963a) reported pancreatic hypertrophy by soybean fractions without trypsin inhibitor activity and Gertler et al. (1967) felt that protease inhibitors played only a minor role in the growth depression occurring with intake of raw soybeans.

Current knowledge indicates that levels and types of protease inhibitors and of other antinutrients, can vary among legume species, varieties and strains (Gupta 1987). Certain other food components such as fatty acids, tannins, lectins, phytates can also combine with trypsin and other enzymes adding to trypsin inhibitor activity (Chan and deLumen 1982, Rackis et al. 1986). The location of inhibitors within soy and other legumes can influence their stability to processing and their potential for interaction and production of synergistic antinutritional effects in animals ingesting them.

ii) Mechanisms of Action - Short-term

Several interrelated mechanisms are involved in the antinutritional and physiological responses of animals to short-term ingestion of plant protease inhibitors. Their occurrence in a given animal reflects the animal's susceptibility to the inhibitor and can be demonstrated by consideration of the sequence of events following ingestion and passage of active trypsin inhibitors into the small intestine of susceptible animal species.

Ingested inhibitors which have not been inactivated as a result of food processing or the acidic conditions of the stomach, form relatively stable complexes with susceptible pancreatic-derived proteases (trypsin, chymotrypsin and elastase) in the small intestine. This initially decreases the level of active proteases and inhibitor-enzyme complexes are lost via the feces (Rackis and Gumbmann 1981, Struthers et al. 1983).

Ingested inhibitors act indirectly to cause pancreatic trophic responses. Their influence occurs in the lumen of the proximal small intestine (Ihse et al. 1977, Schneeman et al. 1977) where complex formation lowers the level of active proteases sufficiently to suppress their

normal feedback inhibition on pancreatic enzyme synthesis and secretion (Green and Lyman 1972, Nitsan and Liener 1976b, Rothman 1986).

The hormone cholecystokinin, a known stimulator of pancreatic trophic response (Struthers et al. 1983) is an intermediary in the process in susceptible animals. Trypsin inhibitors, and other dietary proteins stimulate the release of CCK from endocrine cells of the intestinal mucosa into the blood and it is the increase in CCK levels that ultimately influence the pancreas. The pancreatic trophic response may be a homeostatic mechanism to prevent pancreatic cell damage by persistence of high CCK concentrations (Schneeman and Lyman 1975, Green et al. 1986).

Trypsin sensitive, CCK-releasing peptides have also been implicated. Miyasaka and Green (1983) suggested that a spontaneously-secreted polypeptide of the intestinal mucosa, normally inactivated by trypsin, serves to stimulate CCK release into the blood when active luminal trypsin is decreased by combination with dietary proteins including trypsin inhibitors. Fushiki and Iwai (1989) proposed that a bioactive peptide (monitor peptide) secreted by the pancreas in response to decreased lumen levels of active trypsin and chymotrypsin similarly stimulates CCK release from the intestinal mucosa with subsequent trophic effects on the pancreas.

Other dietary components such as peptides, amino acids, triglycerides, fatty acids and oligosaccharides also increase blood levels of CCK and pancreatic stimulus in certain species (Meyer and Jones 1974, Meyer et al. 1976). Proteins including soybean trypsin inhibitors, however, appear to be the major factor in rats (Temler et al. 1984, Liddle et al. 1986). The rapidly increased synthesis and release of digestive enzymes by the exocrine pancreas occurring with ingestion of trypsin inhibitors in susceptible species, is accompanied in the short term, by a number of pancreatic cellular changes. Electron micrographic studies by

Oates and Morgan (1984) indicated initial depletion of zymogen granules, destruction of cell organelles and formation of vacuoles within hours of ingestion of raw soy flour by 3-month-old male Wistar rats. Rapid increase in pancreatic weight and RNA content was followed by prolonged increase in pancreatic protein content and secretion of pancreatic enzymes. A biphasic pattern of increased DNA synthesis was proposed to initially involve a regenerative response of acinar cells to necrosis induced by the raw soy, followed by a second more prolonged synthesis by both ductal and acinar cells as a result of the continued trophic stimulation of the raw soy. Both hypertrophy (increase in cell size) and subsequent hyperplasia (increase in cell number) persisted over the 28 days of the study.

Increases in pancreatic DNA, RNA and protein levels and in levels and activity of proteases, occurring in rats and other species fed trypsin inhibitors either in pure form or present in raw soy or other legumes, have amply confirmed the influence of trypsin inhibitors on hypertrophy and hyperplasia (Crass and Morgan 1981, Struthers et al. 1983, Temler 1980, Temler et al. 1984a). Light and electron microscopy studies have revealed increased numbers and size of zymogen granules, increased islets of Langerhans cell size, β-cell proliferation, and increased β-cell mitotic figures following colchicine stimulation, in pancreas of weanling and older rats fed raw soy flour over 30 days. Only 7 days exposure to purified soybean trypsin inhibitor in drinking water or 7 days of intraperitoneal injection of CCK was required to produce similar results (Yanatori and Fujita 1976, Folsch et al. 1974).

The reductions in protein digestibility, amino acid availability and growth deficit resulting from trypsin inhibitor ingestion are intimately related to the changes already described. Binding of proteinases in inhibitor-enzyme complexes lowers proteolytic activity resulting in reduced digestibility of dietary protein in many species (Struthers et al. 1983a). Proteolysis of endogenous proteins such as proteases is also compromised and a net deficit

in nitrogen absorption occurs as demonstrated by in vitro decrements in nitrogen absorption in gut segments of rats fed raw compared to heated soy diets (Carroll et al. 1952). Decreased proteolysis likely contributes to the increased intestinal and fecal levels of proteolytic enzyme (Nitsan and Liener 1976b) resulting from continued pancreatic response.

Studies using chickens (Bielorai et al. 1972, 1973) suggested that the net reduction in protein digestibility in this species stems from a reduction in active proteases in the gut distal to the duodenum (or ileum) and decrease in post ileum breakdown of proteins of endogenous origin.

The inherent resistance or refractory nature of soy protein to protease digestion has been proposed as an additional factor contributing to the poor utilization of raw soy in several species (Kakade et al. 1974, Liener 1976). The fact, however, that trypsin inhibitors added to diets containing free amino acids or hydrolyzed protein also inhibit growth in rodents (Westfall et al. 1948, Liener and Fevold 1949), indicates that additional mechanisms are involved.

Several studies have demonstrated that trypsin inhibitors also depress the availability of essential amino acids. In vitro release of all amino acids was inhibited by trypsin inhibitors (Almquist and Merritt 1953) and delayed absorption, lower apparent digestibility of all amino acids and lower net absorption of all but sulfur amino acids were shown in rats fed raw versus heated soy flour (Nitsan and Liener 1976a). Booth et al. (1960) had previously found that combined addition of four essential amino acids (methionine, tyrosine, threonine and valine) was effective at overcoming the poor growth and reduced feed efficiency but not the pancreatic hypertrophy seen in rats fed a raw soybean meal-based diet. Heating of the raw soybean meal prevented both the growth and feed efficiency deficits and pancreatic hypertrophy. It was suggested that the trypsin inhibitors present in raw soy meal stimulated

the pancreas resulting in excess loss of critical pancreatic enzyme amino acids via the feces. Amino acid supplementation overcame the amino acid loss but not the pancreatic stimulation whereas heating destroyed the trypsin inhibitors preventing both the pancreatic stimulation and the excess amino acid loss. Yen et al. (1974) similarly proposed that the inability of supplementary methionine to overcome growth failure and lack of pancreatic response in swine fed trypsin inhibitors indicated unavailability of all dietary amino acids required for protein synthesis.

The influence of dietary trypsin inhibitors on sulfur amino acids are a major area of interest. Depressed protein digestibility has been proposed to result in delayed release or decreased availability of dietary sulfur amino acids. The low sulfur amino acid levels in legumes can add to the problems of sulfur amino acid availability and the loss of cysteine-rich proteases with formation and loss of enzyme-inhibitor complexes can increase sulfur amino acids requirements. Preferential synthesis of pancreatic proteases may be involved in the body growth deficits often observed and the utilization of cystine in their synthesis may prevent any sparing effect of cystine on methionine requirements (Melnick et al. 1946, Kwong et al. 1962, Kwong and Barnes 1963, Frost and Mann 1966, Liener 1976). A specific block in the utilization of cystine because of trypsin inhibitors has also been proposed, and this could upset optimal endogenous ratios of methionine and cystine for growth (Kwong and Barnes 1963, Shannon et al. 1972).

Nitsan and Liener (1976a) reported reduced carcass retention of cystine in rats fed raw versus heated soy flour, although apparent digestibility and absorption of methionine and cystine from raw soy was equivalent or better than in rats fed heated flour. Earlier studies demonstrated marked unavailability of cystine in unheated compared to heated navy bean trypsin inhibitors fed as cystine supplements to chicks. Reduced growth, increased fecal

levels of protein-bound cystine and other amino acids and reduced intestinal protease levels in chicks fed raw navy bean inhibitor indicated that resistance of the navy bean trypsin inhibitor to digestion was a major factor in the lower availability of cystine. This was supported by resistance of the unheated inhibitor to in vitro protease digestion (Kakade et al. 1969).

Sulfur amino acid supplements in some species appear to play a role in both the antinutritional and pancreatic trophic response to trypsin inhibitors. Methionine supplementation of a diet high in SBTI but otherwise adequate for maximal rat growth, suppressed most of the antinutritional effects of the inhibitor. Pancreatic trophic response however, still occurred (Temler et al. 1984a). Similarly, chicks fed methionine-supplemented raw soy (high in trypsin inhibitors) had improved growth but even greater increase in pancreatic weights compared to chicks fed raw soy without added methionine (Bajjaleh et al. 1980). It was suggested that this reflected amelioration of methionine deficit, permitting increased pancreatic cystine synthesis with its incorporation into pancreatic enzymes resulting in increased intestinal proteolysis.

The greater deficits in growth, higher pancreatic weights and poorer feather development of goslings compared to chicks fed raw soy (Nitsan and Nir 1977) further point to the intimate involvement of sulfur amino acid availability. Higher cystine requirements of the normally more heavily feathered goslings exacerbated the growth, pancreatic and feathering response to the dietary trypsin inhibitors present in the raw soy. According to Liener (1976) the growth depression observed in young animals results from the diversion of dietary sulfur amino acids from body tissue synthesis to synthesis of pancreatic enzymes. Barnes and Kwong (1965) showed increased pancreatic conversion of radio-labelled methionine to cystine and increased intestinal and fecal levels of labelled cystine in rats after

a single intragastric dose of soy trypsin inhibitor. They concluded that this increase indicated greater synthesis and release of cysteine-rich proteinases and their subsequent loss via the feces. They also suggested that the higher proportion of protein-bound labelled cystine found in the lower gut and feces of rats fed penicillin and raw soy flour reflected cystine protection from bacterial hydrolysis and corresponded to higher levels of active proteases in feces. The increased synthesis of cystine-rich proteases, their inactivation and/or subsequent loss via the feces as also suggested by results of other workers (Liener 1962, Struthers et al. 1983a), could accentuate the low dietary supply of first limiting sulfur amino acids in soy protein.

The depression in fat absorption occurring with ingestion of raw soy may also involve the influence of trypsin inhibitors. Sklan et al. (1973) showed that raw soy meal accelerated the secretion of fatty acids, lipid phosphorous, cholesterol and other bile components into the duodenum and decreased their absorption. Isolated soy trypsin inhibitors and soy protein fractions causing pancreatic hypertrophy, also caused gall bladder contraction, increased bile output and decreased fat absorption (Sambeth et al. 1967, Rackis 1974). Roy and Schneeman (1981) similarly showed enhanced bile acid secretion in mice fed trypsin inhibitors. Binding of bile acids by undigested proteins of raw soy may cause reductions in fat absorption.

Rackis and Gumbmann (1981) suggested that the poor fat absorption, depressed metabolizable energy and changed carbohydrate metabolism in rats and chicks fed raw soybeans were interrelated. Induced compensatory pancreatic reactions and general stimulating effects on other endogenous secretions resulted from the proteolytic inhibition caused by trypsin inhibitors and contributed to the other antinutritional effects.

It appears that the interrelated effects of limited proteolytic activity with ingestion of raw soybeans containing trypsin inhibitors, the resistance of raw soy protein to proteolysis

and the enhanced amino acid requirements for increased synthesis of pancreatic proteins and secretion of pancreatic enzymes all contribute to the antinutritional effects observed.

b) Long-term Carcinogenic Effects

The work of McGuinness et al. (1980, 1984) indicated that long-term feeding of full fat soy flour to rats not only maintained the pancreatic trophic response seen in the short-term, but also resulted in development of pancreatic nodules, an exponential increase in both pancreas size and weight and in some cases pancreatic and liver cancers. Both raw and heated soy flour diets also potentiated the development of azaserine-induced pancreatic cancer in rats. The combination of dietary soy with exposure to azaserine, was associated with lower effective azaserine dose levels, decreased onset time, increased numbers of multiple nodules and increased incidence of pancreatic cancer (McGuinness et al. 1984). Raw soy flour similarly enhanced the weak carcinogenic effects of the nitrosamine derivative di(2-hydroxypropyl)nitrosamine in rats (Levison et al. 1979).

The United States Department of Agriculture (USDA)-Trypsin Inhibitor Study showed that the incidence of nodular hyperplasia and pancreatic acinar cell adenoma occurring in rats fed soy-based diets over 2 years, was positively associated with the level of dietary trypsin inhibitors (Gumbmann et al. 1985, Liener et al. 1985, Rackis et al. 1985, Spangler et al. 1985). These chronic studies also indicated interaction between dietary protein and trypsin inhibitors.

In the USDA study, soy-based diets containing different levels of protein and varying in SBTI content were fed to male Wistar rats for up to 2 years. Pancreatic hypertrophy and hyperplasia associated with dietary trypsin inhibitor levels, were evident by 6 months as

indicated by increased pancreatic weight, RNA and DNA. Hyperplasia increased with time of exposure to the SBTI. The incidence of pancreatic nodules, grossly visible at 15 months, was highly correlated with dietary SBTI level. Of three dietary protein levels tested (10, 20, 30% of diet), the 20% level resulted in highest incidence of pancreatic nodules (Liener et al. 1985).

Microscopical observations indicated that a generalized hypertrophy of pancreatic tissues occurred in response to prolonged intakes of soy trypsin inhibitors with multiple foci of pancreatic parenchyma undergoing hyperplasia. These foci, whose incidence was related to duration of exposure and level of dietary trypsin inhibitors, involved pancreatic acinar cells. The nodules included a low occurrence of benign adenomas and a higher incidence of neoplasia. No carcinomas were observed (Spangler et al. 1985).

Both soy protein isolates and raw soy flour induced development of nodular hyperplasia and acinar cell adenoma, dependent upon the level of trypsin inhibitors they contained (Gumbmann et al. 1985). This finding would suggest that the non protein components of raw soy flour did not contribute significantly to the abnormal pancreatic histology that occurred with long-term ingestion.

i) Species Differences

As was seen in the short-term, the susceptibility of different animal species to long-term effects of SBTI appears to vary. Gumbmann et al. (1989) found similar short-term responses of mice and rats to trypsin inhibitor concentrates from raw soy and potato. Decreased weight gains, pancreatic hypertrophy and lower apparent nutritional quality occurred when the two concentrates were added to diets fed to both species. After 95 weeks, mice appeared resistant to the trypsin inhibitor dose-related development of nodular hyperplasia and acinar cell adenoma that occurred in the rats. It would appear that the short-term response of a species to dietary trypsin inhibitors, may not be predictive of long-term susceptibilities.

Liener and Hasdai (1986) reported pancreatic enlargement but a low incidence of atypical acinar cell nodules in mice fed raw soy for 18 months. Mice were also resistant to the combined carcinogenic effects of raw soy and azaserine. Hamsters fed a diet of raw soy for 15 months, showed no pancreatic enlargement and a low incidence of tumors caused by injection of a known carcinogen, N-nitrosobis (2-oxypropylamine) (BOP) in combination with the dietary raw soy. Heated soy flour diets combined with BOP injections, however, resulted in an 88% incidence of pancreatic tumors in the hamster. Ausman et al. (1985) fed soy protein isolate for up to 4 years to cebus monkeys. No abnormalities, including biochemical and histological changes of the pancreas were observed, although the SBTI levels of the soy protein isolate fed were low compared to those used in the USDA study. The earlier studies using rats, mice and hamsters suggest that extrapolation from one species to another in terms of carcinogenic potential of SBTI requires caution.

Gumbmann et al. (1989) proposed that the factors involved in species differences in susceptibility to dietary trypsin inhibitor-induced pancreatic lesions reflected: variation in the capacity of specific inhibitors to activate any pancreatic regulatory feedback system (governed by the capacity of the inhibitors to interact with the species proteolytic enzyme profile); differences in the nature and extent of reversible adaptive pancreatic response to the diet (occurrence of hypertrophy and hyperplasia); and variation in species propensity for neoplastic transformation with increased hormonally-stimulated cellular proliferation. As earlier described, the first two components also influence short-term antinutritional responses to dietary trypsin inhibitors.

ii) Protective Effects

Human epidemiological evidence suggests that legume-rich diets play a protective role against human cancer. Seventh-Day Adventist vegetarians were reported to have a lower incidence of cancer than the general population (Phillips 1975) and high intakes of cereals and legumes have been correlated with lower incidence of breast, colon and prostatic cancers (Armstrong and Doll 1975). International correlation studies also reveal a strong negative correlation between dietary caloric contribution of vegetables (especially beans) and incidence of colon cancer (Correa 1981). Troll et al. (1986), on the basis of various epidemiological studies and experimental evidence, suggested that any induction of pancreatic cancer caused by SBTI was species specific and that humans were not susceptible.

Experimental results showed that several synthetic and naturally occurring protease inhibitors were effective at inhibiting in vitro transformation or in vivo tumor promotion. Nanogram quantities of pure BBI blocked the in vitro transformation of C3H/10T1/2 cells

induced by ionizing radiation at the initiation stage of carcinogenesis (Yavelow et al. 1983) and soy KTI suppressed the promotion stage of radiation transformation (Kennedy and Little 1981).

Animal studies have demonstrated that SBTI can have a protective role against certain cancers. Troli et al. (1980) found a lower incidence of x-irradiation-induced mammary cancer in Sprague Dawley rats fed raw soy-based diets, high in trypsin inhibitors, compared to similarly irradiated rats fed low inhibitor-containing chow or casein-based diets. Becker (1981) also reported a lower incidence of spontaneous liver cancer in mice fed diets high in SBTI.

iii) Mechanism of Action - Long-term

The mechanisms involved in both neoplastic transformation and the protective effects resulting from long-term ingestion of soy products containing trypsin inhibitors, have not been defined. Several hypotheses have been proposed.

Carcinogenesis is usually divided into initiation and promotion (or post initiation) stages. Nuclear DNA damage occurring in cells during initiation is considered the primary carcinogenic event and selective factors or influences occurring during the post initiation stage have the potential to stimulate initiated cells via a process called promotion (Farber 1984). There are thus two stages during which soybean trypsin inhibitors could influence carcinogenesis.

The same mechanisms of pancreatic stimulation that occur in the short-term appear to be involved in the long-term. A major feature is the increased release of cholecystokinin, its continued elevated blood concentration and the resultant trophic response of the pancreas.

The stimulation of pancreatic cell division and DNA synthesis resulting from trypsin inhibitors has been previously described as biphasic and ongoing in the rat (McGuinness et al. 1984). There are also indications that this stimulation is reversible with removal of the stimulus and that meal feeding or intermittent exposure to trypsin inhibitors can accentuate the response (Morgan et al. 1986).

Rapidly dividing cells are especially sensitive to the effects of carcinogens (Ryser 1971) and SBTI-induced hyperplasia may render pancreatic cells more susceptible to initiation by chemical factors. Alternatively, increased cell divisions may increase the probability of cell transformation. Roebuck (1986) suggested, the acinar cell foci, (the regions of nodular hyperplasia described in the USDA study) represent populations of preneoplastic cells from which adenomas and adenocarcinomas can develop. It is interesting to note that increased cell replication is a key event in the progression of chemically induced cancers of the liver and other tissues (Farber 1967, Tatematsu et al. 1983).

The cancer promoting and protective effects of SBTI, may also be related to their influence on amino acid availability. Limited methionine or cysteine availability may decrease the synthesis of glutathione, a compound known to be involved in detoxification of carcinogens (Meister 1988). Decreased glutathione synthesis has also been implicated as a means of decreasing inactivation of cancer drugs, thereby increasing their effectiveness (Waxman 1990).

The post initiation stage may also be susceptible to influence by trypsin inhibitors. Decreased digestibility of dietary or endogenous proteins may decrease availability of amino

acids for rapidly dividing tumor cells. This decrease may serve to limit tumor growth or prevent successful proliferation of initiated cells.

Other dietary factors such as protein, selenium, vitamins C, E and A and retinols have often been associated with protection against carcinogenesis. Increased levels of hepatic microsomal enzymes, enhanced glutathione peroxidase activity or improved antioxidant status resulting from their intake have been proposed to increase the metabolism, inactivation or clearance of xenobiotics or to enhance inactivation of free radicals and lipid hydroperoxides generated by toxicants (Newberne and Conner 1986). Some dietary factors have been linked to a higher incidence of certain cancers in man and other animals (Hiryama 1979, Birt et al. 1979). In some cases, factors previously described as protective have been implicated in potentiation. The high unsaturated fat levels of the soybeans and of several soy-based diets used in long-term carcinogenic studies have been shown to contribute to trypsin inhibitor activity and to have a promoting effect on pancreatic carcinogenesis in experimental animals. Refractory, undenatured protein present in soy may also bind to trypsin increasing CCK levels and pancreatic trophic response.

In the USDA rat study, the development of nodular hyperplasia and acinar cell adenoma was directly correlated with trypsin inhibitor levels including those of raw soy protein isolate fed in semi-purified diets. Roebuck et al. (1987) similarly, found that raw soy protein isolate had a significantly greater positive influence on the growth of pancreatic lesions in young male rats initiated with a single dose of azaserine than did unsaturated fats. Raw soy protein also induced a prolonged plasma elevation of the pancreatic trophic effector CCK. The absence of CCK stimulation and the lower carcinogenic influence of the unsaturated fat, suggests the two dietary factors may effect pancreatic carcinogenesis by different mechanisms.

The development of liver cancer in rats and other animals fed methyl deficient diets with and without chemical carcinogens (Mikol et al. 1983, Ghoshal and Farber 1984) indicates another possible area in which trypsin inhibitors may play a role. Methyl deficit can derive from insufficient dietary supply of a number of factors called lipotropes which include methionine, choline, vitamin B_{12} and folate. Lipotropes are essential for many metabolic processes, including normal lipid metabolism, maintenance of membrane integrity, cell proliferation, and synthesis of DNA and nucleoproteins, and their dietary deficit has been shown to influence the development of many forms of cancer (Rogers and Newberne 1975, Rogers and Newberne 1980).

The mechanism(s) involved in the initiation or potentiation of liver cancers by lipotrope or methyl deficiency is unkown; it may operate at several levels. Alterations in DNA caused by generation of free radicals and lipid peroxidation (Rushmore et al. 1984, 1986), hypomethylation of specific genes (Hoffman et al. 1984), membrane damage and decreased levels of drug metabolizing enzymes and reduced immunocompetence (Newberne 1986) have been implicated.

The transmethylation-transsulfuration pathway is of major importance in the metabolism of sulfur amino acids. It involves a series of enzymatically catalyzed steps in which methionine is activated, its methyl group is transferred to one of a number of important compounds, and its sulfur atom is transferred to serine eventually resulting in the synthesis of cysteine and taurine (Finkelstein 1990).

The initial reaction of the transsulfuration pathway involves the generation of S-adenosyl-L-methionine (S-adenosylmethionine, SAM), a high energy sulfonium compound that is a methyl group donor for a variety of reactions catalyzed by methyl transferase enzymes. Transmethylation reactions, which are thus intimately connected to transsulfuration, give rise

to compounds such as creatine, N-methylnicotinamide, methylhistamine, phosphatidyl-L-choline, melatonin, anserine, epinephrine, ergosterol and some purines and alkaloids (Meister 1985, Cooper 1983).

Various authors have shown the influence of lipotrope deficits or deficiencies in methyl groups on the transmethylation-transsulfuration pathways. These deficits can be induced by dietary restriction of lipotropes or by chemical means which either increase the demand for transmethylation -transsulfuration products or block their synthesis (Shivapurkar and Poirier 1983, Henning et al. 1989).

Reductions in SAM and changes in the methylation index or ratio of SAM to S-adenosyl-L-homocysteine (SAH) have been shown to result. SAH is a potent inhibitor of most methylation reactions and the ratio of SAM/SAH gives an indication of the availability of methyl groups for the various transmethylation reactions (Hoffman et al. 1978).

The influence of SBTI on amino acid availability and more specifically on sulfur amino acid availability or requirements, may affect the transmethylation-transsulfuration pathway and the levels of its components. These effects could have an important influence on the availability of methyl groups and may be a key factor in the mechanism of SBTI-influenced carcinogenesis. Because of the importance of the multitude of compounds that result from methylations mediated via SAM, it seems likely that decreased availability of methyl groups or lipotrope deficit, could also be an underlying mechanism of many of the short-term antinutritional and physiological effects of trypsin inhibitors.

The known protective effects of SBTI for specific cancers may involve similar mechanisms. Troll et al. (1986) suggested that the high amino acid requirements of tumor tissues may be limited by BBI binding of chymotrypsin and resultant reduction in protein digestion and amino acid availability. Unavailability of specific amino acids (leucine, tyrosine

and phenylalanine) because of chymotrypsin binding was also suggested as potentially inhibiting production of active oxygen species by stimulated neutrophills. This inhibition could limit potential DNA damage and transformation.

It was previously described that endogenous trypsin inhibitor levels increase with vitamin deficiency and cell breakdown in animals. These inhibitors, which are not plant-derived, also increase in response to emphysema and inflammatory reactions in man and to the increased proteolytic activity associated with in vitro transformed cells. As also previously described, trypsin inhibitors can also potentiate the effects of chemical carcinogens, or by themselves initiate cancers in animals. Rackis et al. (1986) suggested that the contradictory protective-enhancing effects of trypsin inhibitors reflect the multispecificity of proteinase inhibitors and their capacity to potentially inhibit tumor specific proteinases and also adversely affect normal proteinase-controlled biological systems inducing a cascade effect in vivo.

c) Human Implications of Dietary Trypsin Inhibitors

The human concerns for dietary trypsin inhibitors can be divided into antinutritional and carcinogenic aspects. Nutritionally, soy protein can adequately meet the protein maintenance requirements of the adult human (Scrimshaw et al. 1983) and the use of soy and other legumes has been recommended as a means of improving lipid status (Erdman and Fordyce 1989). Soy-based infant formulas have widespread acceptance and are effective nutritional alternatives for human infants (Wink 1985). It has been demonstrated in vitro that human pancreatic trypsin and chymotrypsin can be completely inactivated by physiologically feasible concentrations of soybean protease inhibitors (Krogdahl and Holm 1979), although proper processing of soy products greatly restricts such possibilities. The role of soy protein and the

SBTI they contain in the promotion and potentiation of pancreatic carcinogenesis in laboratory animals, however, is of concern.

Pancreatic cancer is the fifth most common cause of cancer in the United States and its worldwide incidence is on the increase (Mack 1982, Morgan and Wormsley 1977). The etiology of human pancreatic cancer is obscure and the study of the influence of SBTI in relation to the human disease has been minimal (Mack 1986). The preceding review has revealed that like susceptible laboratory animals, the human does appear to have a feedback mechanism controlling pancreatic output. Duodenal infusion of isolated BBI has been shown to be effective at increasing the activity and output of pancreatic enzymes in healthy subjects (Goodale et al. 1985, Liener et al. 1988) and human proteases have been shown, in vitro, to be susceptible to inactivation by inhibitors (Feeney et al. 1969).

Human intake of protease inhibitors from both plant and animal food sources can be considerable (Doell et al. 1981) and certain risk groups may be exposed to higher amounts of dietary protease inhibitors than the general population. The rapidly growing human infant, with both high amino acid requirements and tissues that are already under rapid cell division may be especially susceptible to any toxic or synergistic effects of SBTI. Obviously further investigations of the mechanisms involved in animal response to soybean trypsin inhibitors are warranted.

III. EXPERIMENTAL SECTION

1. TRYPSIN INHIBITOR LEVELS IN SOY-BASED INFANT FORMULAS AND COMMERCIAL SOY PROTEIN ISOLATES AND CONCENTRATES

INTRODUCTION

Soy-based infant formulas and foods are important dietary components for many children. Soy-based human milk substitutes represent about 25% of liquid formulas sold (Ausman et al. 1986) and can be the sole source of nutrition for certain infants at a time of rapid growth and cell division. In addition, the use of soy proteins in processed foods for functional or nutritional purposes has been increasing (Waggle et al. 1990).

Naturally occurring trypsin inhibitors in soy products contribute to growth depression, increased secretion of pancreatic enzymes, lowered protein digestibility, and pancreatic hypertrophy and hyperplasia in several species (Rackis and Gumbmann 1987). In long-term rat feeding studies these inhibitors also cause pancreatic adenoma and acinar cell carcinoma (Gumbmann et al. 1985). The human health implications of trypsin inhibitors are also of concern (Roebuck 1987, Wormsley 1988).

Despite inactivation during processing, up to 20% of trypsin inhibitor activity can remain in soy products (Rackis et al. 1986). Doell et al. (1981) determined trypsin inhibitor concentrations in common foods. Levels in a few commercial concentrates and/or ready-to-feed forms of soy infant formulas have also been reported (Theuer and Sarett 1970, Churella et al. 1976). Recent modifications of the enzymatic assay for trypsin inhibitor activity and

new formulations and methods of processing suggested that it would be worthwhile to measure trypsin activities in soy-based infant formulas. This paper reports levels of trypsin inhibitors in different forms of currently available infant formulas, and in samples of raw soy beans, soy protein isolates and concentrates using two modifications of the standard Kakade et al. (1974) enzymatic assay for trypsin inhibitors.

MATERIALS AND METHODS

Infant formulas in ready-to-feed (RTF), concentrate (CON) and powder (PF) forms were purchased from local pharmacies. Liquid concentrates and ready-to-feed forms were pooled on the basis of brand name and preparation type in stainless steel trays, frozen and lyophilized using a Virtis 12 Freeze Drier (Virtis Corporation, Gardiner, NY). Resultant dry samples were ground to a fine powder (0.5 mm screen) using a Retsch Mill (Retsch KG, Haan, West-Germany). Raw, seed grade, soybeans purchased from a local producer, and commercially available soy protein isolates and concentrates and powdered infant formulas were also ground and freeze-dried. Excessive heating of samples was avoided by controlling rate of passage through the mill.

Fat was extracted from 2 g samples of each product to avoid potential interference by free fatty acids in the trypsin inhibitor assay (Wang et al. 1975). A Goldfish apparatus (Labconco Corp., Kansas City, MO) using diethyl ether as solvent and a 4-hour extraction time permitted simultaneous extraction of 6 samples. The lyophilized, fat-extracted samples were stored at -70°C until analyzed for nitrogen content and trypsin inhibitor levels.

Nitrogen was determined in duplicate using a Kjeltec nitrogen analyzer (Tecator Instruments, Hoganas, Sweden). Crude protein was calculated by multiplying percent

nitrogen by the factor 6.25.

Two modifications of the trypsin inhibitor assay method of Kakade et al. (1974) were used. A slight modification of the method as described by Smith et al. (1980), involved suspending freeze-dried, defatted samples in 0.01 M NaOH (0.50 g in 50 mL) with pH adjustment (9.4-9.6) and stirring for 3 hr using a multiplace stirring table. Gentle shaking and the action of a small stirring bar present in each parafilm-covered extraction flask overcame any initial clumping of samples. After extraction, sample volumes were made up to 100 mL with distilled water, and further diluted to give 40-60% inhibition of the trypsin standard when measured in the assay. Freshly prepared Nα-benzoyl-DL-arginine-p-nitroanilide (BAPNA) hydrochloride solution (Sigma Chemical Co., St. Louis, MO) (0.40 g dissolved in 1 mL dimethyl sulfoxide) diluted to 100 mL with prewarmed Tris (50 mM, pH 8.2 containing 20 mM calcium chloride) was kept at 37°C. Sigma Type IV bovine trypsin (0.040 g in 2 L of 1 mM HCl) and 30% acetic acid solutions were prepared weekly. Triplicate tubes of reagent blank (a), standard (b), sample (c) and sample blank (d) were prepared as described by Smith et al. (1980). Reagent additions, reaction times and absorbance measurements at 410 nm were carried out as described and trypsin inhibitor levels were determined according to the formula:

$$TI \text{ (mg pure trypsin inhibited per g)} = (2.632 \times D \times A_1) / S \text{ or } P$$

where:

D = dilution factor not including original 50 mL dilution

$A_1 = (\text{Absorbance}_b - \text{Absorbance}_a) - (\text{Absorbance}_c - \text{Absorbance}_d)$

S = weight of original sample

P = weight of protein in sample

Separate extractions of the defatted samples followed the method of della Gatta et al. (1988). Samples (0.50 g) were stirred for 2 hr in 200 mL glycine buffer (pH 11) containing 0.01 N each of glycine, sodium chloride, sodium hydroxide, urea and EDTA. Triplicate aliquots (0.2 to 2 mL) of the extract were made up to 2.0 mL with glycine buffer. Subsequent analysis was similar to that for the NaOH extracts but used diluted samples giving 10-70% inhibition of standard for calculation. To compare the effectiveness of the glycine versus the NaOH extraction methods, reagent blanks, sample blanks, standard and samples were also measured and the same formula used for calculation.

Student's t-test (Snedecor and Cochran 1967) was used to compare results for the two extraction methods for each sample and where possible, to compare trypsin inhibitor levels in the different forms of the various infant formula products.

RESULTS

Raw ground soybeans had the highest trypsin inhibitor levels (Table 1.1) although values were much lower following the 2-hr glycine-buffered extraction compared to the 3-hr NaOH extraction. The soy protein isolates and soy concentrates had from 3 to 8% and 15 to 20% of the activity measured in the raw Maple Arrow soybeans after NaOH extraction. Trypsin inhibitor activity of isolates and concentrates were usually also lower following the glycine compared to the NaOH extraction method.

Trypsin inhibitor levels in the infant formulas tested (Table 1.2) were low relative to levels in raw soy and usually similar to those measured in soy protein isolates. Exceptions were the ready-to-feed products RTF #5 and RTF #6 which had 29% and 13% of the trypsin inhibitor level measured in the raw Maple Arrow soybeans after NaOH extraction. In general,

for a given product, the ready-to-feed form had higher activity than powder or concentrate forms. This was demonstrated following both NaOH and glycine buffer extractions. RTF #3 and RTF #4 had the lowest trypsin inhibitor levels of the ready-feed products.

Glycine-buffered extracts of ready-to-feed forms of infant formula often had lower trypsin inhibitor levels compared to NaOH extracts and this was especially evident for RTF #5 and RTF #6. A similar pattern was seen for concentrates and powder forms.

DISCUSSION

Trypsin inhibitor activity was detected in all soy products tested. The variation between different protein isolates and concentrates likely reflects differences in processing and in levels of more heat-resistant types of trypsin inhibitors present in the raw soy sources from which they derived.

Theuer and Sarett (1970) reported wide variation in residual trypsin inhibitor levels in three commercial concentrates of soy isolate-based infant formulas. Variations were from 1 to 20% of the level found in a soy protein isolate also tested. Inhibitor levels in infant formula concentrates measured in the current study were similar to their values, although levels in the soy protein isolates currently tested were much lower than in the one isolate they tested. This may reflect improvements or changes in current processing methods or perhaps use of a variety of soybeans with lower levels of trypsin inhibitors. Trypsin inhibitor levels in raw soy samples, soy protein isolates and concentrates, similar to those found here, have previously been reported (Rackis et al. 1985, Ausman et al. 1986).

Churella et al. (1976), using a version of the NaOH extraction method, found that trypsin inhibitor levels in concentrate and ready-to-feed forms of soy-based infant formulas

ranged from 6 to 15% and from 11 to 56%, respectively, of the level in a soy protein isolate tested. These same authors reported that resterilization had little effect on the low levels of activity present in two commercial infant formulas.

The infant formula concentrates tested here, were considerably lower in trypsin inhibitor activity than corresponding ready-to-feed forms, also suggesting processing differences during preparation. The ready-to-feed products, RTF #3 and #4 were commercially-prepared dilutions of manufactured liquid concentrates that had been heat processed, rebottled, and nippled for easy use in hospital nurseries. These two resterilized products had the lowest trypsin inhibitor levels of the ready-to-feed forms tested following NaOH extraction, reflecting the fact that they were based on liquid concentrates.

Lower protein quality and digestibility of liquid concentrate versus powdered forms of cow's milk-based infant formulas fed to rats have been reported by Sarwar et al. (1989b). Nutrient availability, as well as trypsin inhibitor activity, could be modified by processing differences in preparation of the different forms of soy-based infant formulas. Because of the extra heating applied, the lower trypsin inhibitor activity of the two resterilized ready-to-feed products, relative to other ready-to-feed forms, therefore might coincide with decreased nutritional quality of these products.

The standard enzymatic method of trypsin inhibitor assay, using NaOH extracts, requires that inhibition of diluted samples range from 40-60% of a trypsin standard under conditions of the assay to avoid problems of nonlinearity of inhibition response (Kakade et al. 1974). The method of della Gatta et al. (1988) is reported to overcome the nonlinearity problem and permits use of sample dilutions giving 10-70% inhibition. The use of this method could eliminate time-consuming repeat analyses often needed to meet the more restrictive inhibition range required when using NaOH extraction. The shorter extraction time could also

reduce total analysis time. Despite, however, the greater volume of glycine versus NaOH buffer used, which might enhance solubility, inhibitor values for several samples were lower when the glycine buffer was used. It would appear that in some cases the 2-hr glycine-buffered extraction did not result in complete extraction of inhibitors. It is suggested that the NaOH extraction method is to be preferred over the glycine buffer extraction method as presently used.

Relatively low trypsin inhibitor levels in soy-based infant formulas compared to levels in other soy products, necessitated concentration of the formulas prior to analysis. Lyophilization in a temperature-controlled system as used in the current study permitted concentration while preventing excessive heating with potential loss of trypsin inhibitor activity. Similarly, the use of the Goldfish fat extraction apparatus with relatively low-boiling ether solvent permitted consistent temperature-controlled lipid extraction.

More sensitive methods for assay of specific protease inhibitors are proving useful for both characterization and quantification of soybean trypsin inhibitors as well as for assay of inhibitors present in other legumes (Brandon et al. 1988, DiPietro and Liener 1989). These methods may prove especially useful in terms of the low levels present in soy-based infant formulas.

Modern soy-based formulas have widespread acceptance and appear to be nutritionally adequate and in some cases to be preferred over cow's milk-based formulas (Ausman et al. 1986, Erdman and Fordyce 1989). Compared to human milk, soy-based infant formulas are lower in methionine + cystine and marginally lower in certain other amino acids. The higher protein content of soy-based formulas, however, means that their nutrient ratings for humans are acceptable (Sarwar et al. 1989a). Churella et al. (1976) also found no evidence of problems in protein or caloric utilization or of pancreatic hypertrophy or hyperplasia in rats fed

diets derived from soy-based infant formulas.

The considerably higher trypsin inhibitor levels of two of the ready-to-use infant formula preparations (RTF #5 and RTF # 6) indicate the potential for high intakes of soybean trypsin inhibitors by infants, especially when fed soy formulas as the sole source of nutrition.

It has been demonstrated in vitro, that human proteases are inactivated by trypsin inhibitors (Feeney et al. 1969). Preliminary studies have also shown that human pancreatic response to trypsin inhibitors is similar to that of animal species susceptible to both potentiation and direct carcinogenic influences of soybean trypsin inhibitors (Goodale et al. 1985, Roebuck 1987, Liener et al. 1988). Human infants undergoing rapid cell division and growth may be especially susceptible to such negative influences. The need for more widespread assessment of trypsin inhibitor levels in soy-based infant food products by government regulatory bodies and for more detailed studies of the influences of trypsin inhibitors from all sources on human physiological responses is warranted

TABLE 1.1 Trypsin inhibitor levels in raw soy and soy protein isolates

Product	NaOH Buffer			Glycine Buffer		
	mg/g	mg/g protein	n	mg/g	mg/g protein	n
Maple Arrow soybeans	25.83±1.53	54.49±3.24	6	16.26±1.33	34.30±2.81[a]	10
Commercial soybeans	26.77±1.49	56.14±3.13	6	16.36±0.32	34.30±0.68[a]	4
Soy protein isolate #1	1.16±0.05	1.44±0.06	4	0.90±0.03	1.11±0.03[a]	4
Soy protein isolate #2	3.64±0.17	4.49±0.21	4	2.88±0.01	3.55±0.02[a]	2
Soy protein concentrate #1	7.30±0.64	11.17±0.98	4	6.26±0.06	9.56±0.10	2
Soy protein concentrate #2	5.40±0.23	8.44±0.37	2	4.36±0.11	6.81±0.17[a]	2
Soy protein concentrate #3	6.00±0.12	9.39±0.18	2	6.14±0.10	9.61±0.16	2

Values are means ± SD

[a] Significantly different from value obtained using NaOH buffer ($P < 0.05$)

TABLE 1.2 Trypsin inhibitor levels of commercial infant formulas

Product	NaOH Buffer			Glycine Buffer		
	mg/g	mg/g protein	n	mg/g	mg/g protein	n
READY-TO-USE FORMS						
Product #1	0.47±0.06	2.56±0.32	4	0.43±0.07	2.35±0.36	4
Product #2	0.64±0.18	3.23±0.89	2	0.52±0.02	2.64±0.11	4
Product #3	0.34±0.01	1.90±0.08	2	0.52±0.01	3.01±0.04[a]	2
Product #4[d]	0.42±0.01	2.09±0.07	2	0.35±0.03	1.77±0.18[c]	4
Product #5	2.72±0.06	15.54±0.33	4	2.09±0.11	12.00±0.64[a]	2
Product #6	1.31±0.02	6.82±0.11	2	0.71±0.01	3.71±0.07[a]	2
CONCENTRATES						
Product #5	0.53±0.08	2.51±0.37[b]	4	0.54±0.04	2.53±0.17[c]	2
Product #6	0.64±0.04	3.20±0.18[b]	4	0.38±0.01	1.91±0.07[ac]	2
POWDERS						
Product #1	0.36±0.04	1.95±0.23[b]	4	0.38±0.08	2.03±0.41	4
Product #2	0.29±0.02	1.73±0.13[b]	4	0.22±0.02	1.32±0.15[ac]	4
Product #3	0.30±0.04	2.20±0.30	4	0.45±0.10	3.31±0.77[a]	4
Product #6	0.59±0.01	3.67±0.08[b]	4	0.43±0.01	2.70±0.09[ac]	2

Values are means ± SD

[a] Significantly different from value obtained using NaOH buffer ($P < 0.05$).

[b] Significantly different from corresponding ready-to-use product using NaOH buffer ($P < 0.05$).

[c] Significantly different from corresponding ready-to-use product using Glycine buffer ($P < 0.05$).

[d] Product #4 is commercially resterilized Product #2.

2. EFFECTS OF SOYBEAN TRYPSIN INHIBITORS AND DL-ETHIONINE ON GROWTH AND SERUM PARAMETERS IN YOUNG RATS

INTRODUCTION

Dietary soybean trypsin inhibitors (SBTI) cause pancreatic exocrine hypersecretion, hyperplasia and hypertrophy in rats and other animals. Formation of inactive inhibitor-protease complexes in the small intestine and resultant loss of normal feedback control of synthesis and release of proteases has been proposed as a cause. Reductions in growth, nitrogen digestibility and Protein Efficiency Ratio are some of the antinutritional consequences of ingestion of SBTI (Green and Lyman 1972, Gumbmann and Friedman 1987, Kakade et al. 1967). Information on short-term changes in clinical chemistry parameters and plasma free amino acids occurring with ingestion of SBTI is limited.

Considerable interest has been shown on the potentiation of pancreatic carcinogens such as di-(hydroxypropyl)-nitrosamine and azaserine by diets containing full-fat raw soy flours (Levison et al. 1979, Morgan et al. 1977) and the potential carcinogenic effects of long-term feeding of SBTI in rats (Gumbmann et al. 1986, Rackis et al. 1985). The mechanism(s) of such effects remain to be elucidated. Deficits, however, in dietary lipotropes (compounds involved in normal lipid and methyl group metabolism) have been implicated in hepatocarcinogenesis in rats both in the presence and absence of chemical carcinogens (Ghoshal and Farber 1984, Mikol et al. 1983, Newberne and Conner 1986, Rogers and Newberne 1975). The sulfur amino acid methionine (first limiting in legumes) is one such lipotrope whose endogenous and dietary supply and utilization are further compromised by the presence of active trypsin inhibitors (Liener and Kakade 1969).

Ethionine, the S-ethyl analogue and metabolic antagonist of methionine, causes acute pancreatitis, renal lesions and a variety of liver changes including necrosis, nucleolar fragmentation, fatty liver and in the longer term hepatocarcinogenesis. Deficits in available methionine appear to be a factor in ethionine carcinogenesis since the normal transsulfuration pathway is compromised with formation of S-adenosylethionine competing with normal methionine activation, resulting in decrease in hepatic levels of S-adenoslymethionine. Decreased ATP, accumulation of S-adenosylethionine and inhibition of normal methyl transfer reactions also occur due to ethionine. Hypomethylation of DNA (normally mediated by S-adenosylmethionine) in liver and testes of rats fed DL-ethionine has also been reported (Shinozuka and Farber 1969, Alix 1982, Kanduc et al. 1988, Shull et al. 1966).

Because of the influence of both SBTI and ethionine on sulfur amino acids, it was of interest to see if short term changes in growth, clinical chemistry parameters and serum free amino acids induced by both might be similar or synergistic. Results could support the hypothesis that lipotrope deficit is a major feature in the carcinogenic mechanisms involving SBTI.

MATERIALS AND METHODS

A basal diet providing 16% crude protein (%N x 6.25) with 8% from a commercial soy protein isolate (Supro 710, Ralston Purina, St. Louis, MO) and 8% from raw ground soy beans (Maple Arrow Soybeans, Hardy Seeds Ltd., Inkerman, Ont.) was supplemented with DL-threonine and DL-tryptophan so as to meet all essential amino acid requirements for rat growth (National Research Council 1978) except for methionine + cystine. The other components of the basal diet are shown in Table 2.1. Determination of the trypsin inhibitor

activity in the two protein sources (Smith et al. 1980) indicated that the basal diet caused an inhibition of 623 mg of trypsin per 100 g of diet. Three other diets, based upon the basal diet had 0.3% DL-ethionine (DL-ETH), 0.9% DL-methionine (DL-MET) and 0.3% DL-ethionine + 0.9% DL-methionine respectively as supplements. Two control diets each providing 16% crude protein were also formulated with ANRC Casein (Humko Sheffield Chemical, Division of Kraft Inc., P.O. Box 630, Norwich, N.Y.) and soy protein isolate (Supro 710) respectively as the sole source of protein. Supplementary DL-methionine, DL-threonine and DL-tryptophan were added to the control diets to ensure equivalency of essential amino acid levels and adequacy in terms of rat growth requirements (National Research Council 1978). Choline bitartrate (0.2%) was also added to each control diet. The soy protein isolate control (SPI control) and casein control diets caused inhibitions of 75 and 0 mg of trypsin per 100 g of diet respectively. Other components corresponded to those of the basal diet. An additional diet based on the soy protein isolate control diet with 0.3% DL-ethionine was also formulated but used only in the trial involving Wistar rats.

The diets were stored in a cold room at 5°C and fed twice weekly in two separate trials. In the first trial, groups of 5 male and 5 female CD Sprague Dawley (SD) rats (108 ± 16 g initial body weight) and in the second, groups of 8 male and 8 female Wistar rats (51 ± 3 g initial body weight) (Charles River Canada Inc., St. Constant, Que.) were fed the various diets and tap water ad libitum. In each experiment, rats were assigned so that the diet groups in each experiment had equivalent mean body weights.

Rats were kept in individual stainless steel, mesh-bottomed cages in an animal room at 24-25 ° C and 50% relative humidity with 12 hour light/dark cycles with light commencing at 7 AM. Body weights and food intakes were measured weekly. The Health Protection

Branch's guide for care and use of laboratory animals (Health Protection Branch 1988) was followed throughout the studies.

During the second week of the test involving Wistar rats, a complete 3-day fecal collection was made from each animal. Feces of rats of the same sex, fed the same diet were pooled and frozen. Nitrogen determinations of feces permitted determination of true protein digestibility using a previously determined value for metabolic fecal nitrogen (Sarwar and Peace 1986). After three weeks, food was removed at 8 AM and animals killed between 9:30 and 10:30 AM under halothane anaesthesia (3% in O_2) by exsanguination from the abdominal aorta. Liver and pancreas of individual rats were quickly removed, weighed and frozen at -70°C. Serum was separated by centrifugation (10 min at 1600 x g). In the case of the Wistar rats, equal volumes of serum from 4 rats of the same sex on the same diet were pooled and deproteinized with 10% trichloracetic acid (1:1 v/v). Sera and pooled deproteinized sera samples (for amino acid analysis) were also stored at -70°C until analysis. Amino acid analysis was by ion exchange chromatography using a Beckman 121 MB Amino Acid Analyzer (Beckman Instruments, Palo Alto, CA). Individual serum samples were also analyzed for cholesterol, triglycerides, urea nitrogen, total protein, glutamic pyruvic transaminase (SGPT), and alkaline phosphatase using an ABA-200 series II Bichromatic Autoanalyzer (Abbott Diagnostics Division, Dallas, TX) and Abbott Clinical Chemistry test kits (Abbott Diagnostics Division, Mississauga, Ont.).

Statistical analysis was carried out using analysis of variance and Duncan's multiple range test (Snedecor and Cochrane 1967, Duncan 1955).

RESULTS

Female rats generally had lower weight gains and feed efficiencies than male rats of the same strain on the same diet (Tables 2.2 and 2.3). For each week of test, gains of female SD rats fed the two control diets were similar. Weight gains of the male SD casein-fed control rats tended to be greater than those of the SPI-fed control males, although this was significant only for the second week. Similarly, feeding both control diets resulted in equivalent weight gains for both male and female Wistar rats except for females during the second week when casein-fed controls had greater gains than corresponding SPI-fed females.

Feeding the basal diet, high in SBTI, resulted in decreased weight gains with male SD rats showing only 20, 64 and 51% ($P<0.01$) of the weight gains of rats fed the SPI control diet for the first, second and third weeks respectively. Female SD rats had better weight gains (53, 79 and 88% for succeeding weeks) relative to female controls while weight gains of Wistar rats fed the basal diet were 56, 60 and 44% (males) and 62, 84 and 65% (females) of the weight gains of their respective SPI-fed controls ($P<0.01$). Feed efficiencies generally reflected weight gains.

DL-methionine supplementation prevented the negative influence on growth and feed efficiency of the SBTI-containing basal diet for both the male and female SD rats. The younger Wistar rats showed improvement with methionine supplementation of the basal diet but weight gains equivalent to those of the controls were not achieved until the second week for the females and until the third week for the males.

Feeding 0.3% DL-methionine in combination with the basal diet, resulted in weight losses and decreased feed efficiencies relative to both the basal-fed and control-fed animals. For both sexes, weight loss and decrease in feed efficiency were greatest during the first week of the test. The male SD rats, for example, lost 27% of body weight during the first week

whereas second and third week losses were each equal to about 6% of body weight. The Wistar rats had similar weight losses. Moreover, during the third week, poor condition and in 3 cases death of animals fed the basal + ethionine diet, necessitated the early termination of this group to ensure samples for clinical analysis.

The effect of DL-methionine supplementation of the basal + ethionine diet on growth performance changed as the experiment progressed. In the first week, weight gains and feed efficiencies of male SD rats were equivalent to those of rats fed the basal diet without ethionine. By the second and third weeks, growth and feed efficiency had further improved to match those of the SPI control rats. Female SD rats fed supplementary methionine in combination with the basal + ethionine diet also had improved weight gains and feed efficiencies although, during the first week of the feeding trial, values were lower than for female SD rats fed the basal diet alone. For male and female Wistar rats, methionine supplementation of the basal + ethionine diet was only partially effective in terms of improved growth. The least improvement also occurred during the first week.

Feeding the SPI + ethionine diet was less damaging in terms of animal growth than was feeding the basal diet with the same amount of ethionine. Weight gains and feed efficiencies were, however, considerably less than those of the control animals.

True protein digestibility (results not shown) on diets fed the Wistar rats was negatively affected by the presence of SBTI. For example, feeding the SPI and casein control diets to male rats resulted in digestibilities of 96 and 97% respectively. Digestibility of the basal-diet was 84% and the ethionine addition caused no additional decrease. Methionine supplementation was not effective in preventing the decreased digestibility.

Final liver weights expressed as a percent of body weight (relative liver weight) tended to be lower for the SPI control-fed versus casein control-fed animals although this was

significant only for the male SD and the female Wistar rats. No differences were seen between relative liver weights of basal-fed and SPI control-fed rats, whereas ethionine addition to the basal and SPI diets resulted in a trend towards increased relative liver weights compared to the SPI control-fed rats.

Feeding the SPI-based, casein-based and basal diets to both strains of rats for three week periods resulted in similar pancreatic weights per 100 g body weight. An increase in relative pancreatic weight compared to control-fed rats was seen with methionine supplementation of the basal diet when ethionine was also present (in the case of male SD and female Wistar rats) and without ethionine (female SD and female Wistar rats). Increases seen with methionine supplementation of other basal diet groups were not significant.

Differences in serum clinical chemistry parameters of the SD and Wistar rats are shown in Tables 2.4 and 2.5 respectively. Serum [illegible] higher in rats fed ethionine-containing diets without supplementary methionine. Serum cholesterol and triglycerides tended to be greater in rats fed the casein versus the SPI-based control diet, although differences were not significant. Methionine added to the basal + ethionine diet prevented any increase in serum triglycerides.

Basal diet-fed rats had higher serum urea nitrogen levels than did rats fed the SPI control diet with the exception of the female SD rats. Urea nitrogen was highest in rats fed the basal + ethionine diet while methionine addition to the basal diet and to the basal + ethionine diet resulted in lower levels.

Supplementary methionine also prevented the high SGPT (measured in Wistar rats only) and alkaline phosphatase levels in rats fed the basal + ethionine diet. The combination of soy protein isolate with ethionine also resulted in lower alkaline phosphatase and SGPT levels compared to those of basal + ethionine-fed rats.

Tables 2.6 and 2.7 show levels of several of the free amino acids in sera of female Wistar rats fed the various diets. Generally similar results were obtained for male rats. Rats fed the two control diets had equivalent levels of serum free essential amino acids (Table 2.6) with the exception of arginine and lysine which were higher ($P<0.01$) in the SPI control-fed rats. Of the non essential amino acids (Table 2.7), glycine and α-aminobutyrate were higher and proline lower ($P<0.05$) in the SPI- versus casein-fed controls.

Addition of ethionine to the basal diet, resulted in higher serum histidine, leucine, and phenylalanine, total essential ($P<0.01$), threonine ($P<0.05$) concentrations and lower arginine ($P<0.01$), tryptophan and lysine ($P<0.05$) relative to the casein control-fed animals. Higher serum glycine, serine, ornithine, taurine and α-aminobutyrate ($P<0.01$) were also observed. Ethionine added to the SPI-based control diet also resulted in higher serum leucine, glycine, ornithine and α-aminobutyrate and lower arginine ($P<0.01$) relative to casein control-fed animals, although differences tended to be less pronounced than in basal + ethionine-fed rats.

Significantly higher serum methionine relative to rats fed the basal and two control diets occurred in rats fed the basal + ethionine + methionine diet ($P<0.01$). Rats fed the SPI + ethionine diet also had significantly higher methionine compared to basal ($P<0.01$) and control ($P<0.05$) fed rats. Methionine sulfoxide, not detected in sera of rats fed either the control or basal diets, was at highest concentration in rats fed the basal + ethionine diet with intermediate levels occurring in rats fed the SPI + ethionine diet and the basal diet with added methionine and ethionine. Methionine sulfone was not detected in the sera of any rats.

Increases in serum histidine, phenylalanine, threonine and total essential amino acids and non essential serine compared to levels in SPI control-fed rats, measured in rats fed the basal or basal + ethionine diets, were prevented by addition of methionine. Higher serum

histidine and phenylalanine and lower arginine, tryptophan and lysine were also prevented or moderated by methionine supplementation of the basal + ethionine diet.

DISCUSSION

The equivalent weight gains and feed efficiencies of rats fed the two control diets indicated the adequacy of the SPI control diet for rat growth. Appropriate DL-methionine supplementation of both diets made them matching in sulfur amino acids. Sulfur amino acid adequacy was further ensured by addition of choline bitartrate which spared methionine requirements for choline biosynthesis (Farber and Ichinose 1956).

The considerable deficits in weight gain and feed efficiency that resulted from feeding the basal diet, were likely caused by both the presence of trypsin inhibitors and the low level of dietary methionine. Up to 50% of the growth deficit of rats fed soy based diets has been attributed to trypsin inhibitors (Rackis 1981). Decreased true protein digestibility, demonstrated in the case of Wistar rats fed the basal diet, likely further contributed to the low availability of first limiting methionine. Decreases in weight gains, Protein Efficiency Ratio and apparent nitrogen digestibility were previously reported in rats fed raw soy flour-based diets (Gumbmann and Friedman 1987). Failure of supplementary methionine to overcome the decreased digestibility of the basal diet or of the basal diet + ethionine fed to the Wistar rats, suggests that dietary trypsin inhibitor levels were sufficiently high to overcome any increased protease synthesis occurring as a result of improved methionine supply.

In the case of the SD rats, the relatively greater deficit in growth parameters during the first week of the feeding trial may be indicative of a greater requirement for sulfur amino acids for growth in younger rats which moderated as the test progressed.

The pancreas is usually the organ of major interest in terms of effects of trypsin inhibitors (Kakade et al. 1967, Gumbmann et al. 1986). Because of the hepatocarcinogenicity of ethionine (Shinozuka and Farber 1969, Kanduc et al. 1988) and because the liver is the major site for amino acid metabolism, it was of interest to look at potential liver changes. In the current study using 16% protein diets, no significant differences in relative liver weights of either the SD or younger Wistar rats fed the basal diet or either control diet were detected after three weeks. Addition of ethionine to the basal diet similarly did not result in any marked increases in relative liver weights. Shinozuka et al. (1978) previously reported increased liver weights and moderate to severe fatty liver in older SD rats fed for up to 30 weeks, a diet deficient in the lipotrope choline. Development of fatty liver because of dietary-induced lipotrope deficit may require a longer time period than the three weeks of the current study.

The fact that higher relative pancreatic weights were not seen in rats fed the basal diet compared to those fed the control diets, despite the known hypertrophic and hyperplastic effects of raw soy and its trypsin inhibitors, might be explained by the relatively high protein levels of both the control and basal diets. Temler (1980) found that high levels of dietary casein and soy protein both contributed to pancreatic hypertrophy in rats.

Significant increases in relative pancreatic weight seen in female SD and Wistar rats fed basal diet with supplementary methionine suggests an additional pancreatic trophic response to the SBTI of the basal diet with improvement in methionine supply. Sharp decrease in relative pancreatic weight seen in SD rats with addition of ethionine to the basal diet suggests a toxic effect that was further indicated by general growth failure and the poor state of the Wistar rats fed the same diet.

Serum urea nitrogen appeared to be the only clinical parameter that changed relative to levels in the control diets with ingestion of the basal diet containing SBTI without ethionine.

Higher serum urea nitrogen in rats fed this diet supports other indications of its lower protein quality (Eggum 1970).

Despite the known cholesterol-lowering effects of soy protein compared to casein (Nagata et al. 1980) no differences were detected in serum cholesterol levels of rats fed the casein control, SPI control or basal diets. Acute injections of ethionine into rats have been reported to result in rapid accumulation of liver lipids (especially triglycerides) (Farber 1963). The increase in serum triglycerides with addition of ethionine to the basal diet may reflect either an initial step in this accumulation or transfer of excess triglycerides to the blood. The normalization of serum triglyceride levels with methionine supplementation of the basal + ethionine diet suggests a deficit in the lipotrope methionine as a factor in the increase.

Increased serum alkaline phosphatase and SGPT that occurred with addition of ethionine to the basal diet suggests damage to liver cells (Tietz 1983). Addition of methionine to the basal + ethionine diet or use of the SPI control + ethionine diet (with low SBTI) resulted in lower serum levels indicating the involvement of methionine deficit in the toxicity of ethionine and suggesting that dietary SBTI enhanced such a deficit.

Serum amino acid differences occurring when the basal diet or diets containing ethionine were fed to the weanling Wistar rats support the idea of a deficit or unavailability of sulfur amino acids because of dietary SBTI and/or DL-ethionine. The more pronounced differences between the basal and casein control rats compared to differences between the basal and SPI control rats likely reflects the 50% contribution of soy protein isolate to the protein of the basal diet.

It has been shown that sulfur amino acid inadequacy relative to growth requirements of young rats, results in poor growth and serum accumulation of essential amino acids (with the exception of methionine) and non essential amino acids related to the transsulfuration

pathway of methionine (Peace et al. 1986). The higher serum levels of essential histidine, leucine, phenylalanine and threonine and total essential amino acids relative to levels in control-fed rats, with the addition of DL-ethionine to basal diet, suggest such an accumulation of essential amino acids, decreased protein synthesis and a deficit in availability of methionine for the transsulfuration pathway. This finding is supported by significant decreases in weight gains seen for both strains of rat when ethionine was present. That SBTI contributed to the deficit is indicated by improved growth and lower levels of most of these amino acids in sera of rats fed the SPI control diet with added ethionine and low SBTI levels.

Accumulation of methionine sulfoxide (oxidized product of unused methionine) and increased serum glycine and serine (unused precursor and substrate for the transsulfuration pathway of methionine) in the sera of basal + ethionine-fed rats, further suggest that the lipotrope methionine is rendered unavailable.

Accumulation of several of the amino acid precursors of normal methionine metabolism that occurred when the basal + ethionine diet was fed, moderated with the addition of methionine or with use of the SPI-based control diet + ethionine rather than the basal diet + ethionine. The high serum levels of methionine and of methionine sulfoxide that were measured in rats fed these diets suggest, however, that methionine availability continued to be compromised by ethionine.

It has thus been demonstrated that feeding diets with high levels of soy trypsin inhibitors to rats of two different strains, resulted in signs of protein inadequacy (poor growth, decreased feed efficiency, reduced true protein digestibility, rise in serum urea nitrogen) which were exacerbated by the addition of DL-ethionine. Higher concentrations of total and of several individual serum free essential amino acids compared to levels in rats fed a casein control diet further suggests reduced protein synthesis. Serum accumulation of amino acid

precursors used in the metabolism of methionine and high methionine sulfoxide levels suggest that both supply and availability of sulfur amino acids are involved in the negative growth and biochemical changes because of dietary SBTI and ethionine. These conclusions are further supported by improvements obtained with methionine supplementation of the basal diet with and without ethionine and by use of a more digestible soy protein isolate-based diet formulated to be adequate in sulfur amino acids. Work is currently in progress to further elucidate metabolic aspects of these changes and to relate them to potential carcinogenic mechanisms.

TABLE 2.1 Composition of basal diet

Ingredients	g/kg Diet
Soy protein isolate[1]	92.6
Soybeans (raw, ground)[2]	213.6
DL-threonine[3]	0.5
DL-tryptophan[3]	0.1
Soybean oil[4]	108.4
AIN Mineral mix[5]	35.0
AIN Vitamin mix[5]	10.0
Corn starch	539.8

[1] Supro 710 (Ralston Purina Co., St. Louis MO).

[2] Maple Arrow Soybeans (Hardy Seeds Ltd., Inkerman Ont.).

[3] Sigma Chemical Co., P.O. Box 14508, St. Louis MO.

[4] Providing 150 g soya oil/kg diet (including oil of raw seeds).

[5] ICN Biomedicals Ltd. St. Laurent, Quebec.

TABLE 2.2 Growth parameters of Sprague Dawley rats fed diets containing soybean trypsin inhibitors and/or DL-ethionine

Diet	Weight gain			Feed efficiency			Liver weight	Pancreatic weight
	Week 1	Week 2	Week 3	Week 1	Week 2	Week 3		
a) Male rats	g/wk			g gain/100 g food intake			g/100 g body weight	
SPI control	46 ± 2^{c}	44 ± 2^{cd}	43 ± 2^{cd}	43 ± 2^{c}	36 ± 1^{c}	33 ± 1^{c}	3.81 ± 0.03^{a}	0.33 ± 0.03^{b}
Casein control	50 ± 1^{c}	54 ± 2^{e}	52 ± 2^{d}	45 ± 2^{c}	41 ± 1^{c}	38 ± 1^{c}	4.52 ± 0.12^{cd}	0.28 ± 0.02^{b}
Basal	9 ± 3^{b}	28 ± 3^{b}	22 ± 5^{b}	10 ± 4^{b}	26 ± 1^{b}	21 ± 4^{b}	4.06 ± 0.15^{a-c}	0.37 ± 0.03^{bc}
Basal + DL-ETH	-31 ± 2^{a}	-5 ± 1^{a}	-5 ± 1^{a}	-89 ± 7^{a}	-15 ± 2^{a}	-15 ± 3^{a}	4.21 ± 0.15^{bc}	0.17 ± 0.04^{a}
Basal + DL-MET	47 ± 3^{c}	49 ± 3de	44 ± 4^{cd}	43 ± 4^{c}	38 ± 1^{c}	33 ± 2^{c}	4.72 ± 0.13^{d}	0.38 ± 0.05^{bc}
Basal + DL-ETH + DL-MET	6 ± 3^{b}	40 ± 3^{c}	37 ± 3^{c}	8 ± 4^{b}	38 ± 3^{c}	34 ± 2^{c}	4.58 ± 0.12^{cd}	0.48 ± 0.05^{c}
b) Female rats								
SPI control	32 ± 1^{d}	29 ± 1^{b}	17 ± 2^{b}	34 ± 1^{cd}	28 ± 1^{b}	18 ± 2^{b}	3.86 ± 0.13^{a}	0.38 ± 0.03^{b}
Casein control	36 ± 2^{d}	25 ± 4^{b}	22 ± 5^{b}	37 ± 3^{d}	24 ± 3^{b}	22 ± 4^{b}	4.31 ± 0.13^{a}	0.39 ± 0.03^{b}
Basal	17 ± 2^{c}	23 ± 6^{b}	15 ± 3^{b}	17 ± 2^{c}	22 ± 5^{b}	16 ± 3^{b}	4.00 ± 0.18^{a}	0.41 ± 0.03^{b}
Basal + DL-ETH	-31 ± 1^{a}	-5 ± 1^{a}	-4 ± 1^{a}	-102 ± 8^{a}	-14 ± 3^{a}	-13 ± 3^{a}	4.18 ± 0.21^{a}	0.11 ± 0.02^{a}
Basal + DL-MET	32 ± 2^{d}	21 ± 1^{b}	18 ± 2^{b}	34 ± 3^{cd}	23 ± 9^{b}	20 ± 2^{b}	4.11 ± 0.19^{a}	0.57 ± 0.08^{c}
Basal + DL-ETH + DL-MET	-2 ± 5^{b}	20 ± 4^{b}	15 ± 5^{b}	-5 ± 10^{b}	28 ± 5^{b}	19 ± 7^{b}	5.26 ± 0.26^{b}	0.44 ± 0.04^{bc}

Means ± SEM for 5 animals per group.

Means of values for rats of the same sex in the same column with different superscripts differ significantly, ($P<0.05$).

TABLE 2.3 Growth parameters of weanling Wistar rats fed diets containing soybean trypsin inhibitors and DL-ethionine

Diet	Weight gain			Feed efficiency			Liver weight	Pancreatic weight
	Week 1	Week 2	Week 3	Week 1	Week 2	Week 3		
a) Male rats	g			g gain/100 g food intake			g/100 g body weight	
SPI control	46 ± 1^{e}	58 ± 2^{d}	59 ± 2^{c}	59 ± 1^{d}	55 ± 1^{e}	49 ± 1^{d}	4.12 ± 0.10^{ab}	0.33 ± 0.02
Casein control	49 ± 2^{e}	56 ± 1^{d}	58 ± 3^{c}	59 ± i^{d}	54 ± 2^{e}	48 ± 2^{cd}	4.23 ± 0.13^{ab}	0.28 ± 0.02
Basal	26 ± 2^{c}	35 ± 2^{b}	26 ± 2^{a}	38 ± 2^{c}	36 ± 1^{b}	27 ± 1^{a}	3.91 ± 0.11^{a}	0.36 ± 0.04
Basal + DL-ETH	-12 ± 1^{a}	-1 ± 1^{e}	-	-40 ± 5^{a}	-2 ± 1^{a}	-	-	-
Basal + DL-MET	37 ± 3^{d}	49 ± 2^{c}	54 ± 2^{c}	53 ± 2^{d}	49 ± 1^{d}	47 ± 1^{bcd}	4.27 ± 0.26^{b}	0.36 ± 0.02
Basal + DL-ETH + DL-MET	12 ± 1^{b}	36 ± 1^{b}	41 ± 2^{b}	23 ± 2^{b}	45 ± 1^{c}	45 ± 2^{bc}	4.45 ± 0.35^{b}	0.38 ± 0.01
SPI control + DL-ETH	10 ± 2^{b}	36 ± 2^{b}	40 ± 1^{b}	21 ± 3^{b}	47 ± 1^{cd}	44 ± 1^{b}	4.36 ± 0.12^{b}	0.32 ± 0.05
b) Female rats								
SPI control	40 ± 2^{e}	38 ± 2^{d}	34 ± 2^{b}	55 ± 2de	43 ± 1^{c}	36 ± 1^{b}	3.80 ± 0.05^{a}	0.36 ± 0.01^{ab}
Casein control	44 ± 2^{e}	44 ± 1^{e}	34 ± 2^{b}	58 ± 1^{e}	44 ± 1^{c}	33 ± 2^{b}	4.44 ± 0.21^{b}	0.36 ± 0.03^{ab}
Basal	25 ± 1^{c}	32 ± 1^{c}	22 ± 1^{a}	37 ± 1^{c}	35 ± 1^{b}	24 ± 1^{a}	4.08 ± 0.12^{ab}	0.40 ± 0.02^{bc}
Basal + DL-ETH	-13 ± 1^{a}	-1 ± 1^{a}	-	-55 ± 6^{a}	-2 ± 2^{a}	-	-	-
Basal + DL-MET	33 ± 1^{d}	39 ± 1^{d}	32 ± 3^{b}	48 ± 1^{d}	43 ± 1^{c}	34 ± 2^{b}	4.27 ± 0.07^{b}	0.47 ± 0.02^{d}
Basal + DL-ETH + DL-MET	12 ± 3^{b}	24 ± 2^{b}	28 ± 1^{b}	26 ± 5^{b}	34 ± 3^{b}	37 ± 4^{b}	4.95 ± 0.21^{c}	0.45 ± 0.01^{cd}
SPI control + DL-ETH	8 ± 2^{b}	30 ± 1^{c}	31 ± 2^{b}	17 ± 3^{b}	40 ± 1^{c}	36 ± 2^{b}	4.52 ± 0.12^{b}	0.33 ± 0.02^{a}

Means ± SEM for 8 animals per group.

Means of values for rats of the same sex in the same column with different superscripts differ significantly, ($P<0.05$).

TABLE 2.4 Serum clinical chemistry parameters of Sprague Dawley rats fed diets containing soybean trypsin inhibitors and/or DL-ethionine

Diet	Cholesterol mg/dL	Triglycerides mg/dL	Urea Nitrogen mg/dL	Total protein g/dL	Alkaline phosphatase IU/L
a) Male rats					
SPI control	84 ± 8	238 ± 31^{a}	13.3 ± 0.5^{ab}	5.9 ± 0.1	496 ± 51^{a}
Casein control	89 ± 12	313 ± 22^{ab}	14.0 ± 0.9^{b}	6.3 ± 0.1	621 ± 73^{a}
Basal	96 ± 9	276 ± 28^{ab}	16.8 ± 0.7^{c}	5.5 ± 0.1	672 ± 56^{a}
Basal + DL-ETH	82 ± 2	465 ± 41^{c}	18.6 ± 0.9^{c}	5.8 ± 0.2	1062 ± 136^{b}
Basal + DL-MET	109 ± 4	365 ± 32^{bc}	10.8 ± 0.7^{a}	6.1 ± 0.1	657 ± 57^{a}
Basal + DL-ETH + DL-MET	75 ± 7	377 ± 36^{bc}	11.7 ± 1.4^{ab}	6 0 ± 0.2	618 ± 46^{a}
b) Female rats					
SPI control	86 ± 4	168 ± 25^{a}	15.6 ± 1.1^{a}	6.6 ± 0.2^{b}	352 ± 48^{a}
Casein control	96 ± 4	240 ± 40^{a}	20.2 ± 1.3^{bc}	6.3 ± 0.1^{b}	413 ± 33^{a}
Basal	80 ± 4	208 ± 19^{a}	16 9 ± 1 2^{ab}	5.7 ± 0.2^{a}	407 ± 44^{a}
Basal + DL-ETH	89 ± 5	340 ± 35^{b}	24.0 ± 2.1^{c}	6.0 ± 0.3^{ab}	719 ± 73^{b}
Basal + DL-MET	85 ± 4	168 ± 24^{a}	15.6 ± 0.9^{a}	6.3 ± 0.2^{b}	316 ± 24^{a}
Basal + DL-ETH + DL-MET	88 ± 11	253 ± 37^{ab}	13.2 ± 1.4^{a}	6 6 ± 0.2^{b}	462 ± 56^{a}

Means ± SEM for 5 animals per group

Means of values for rats of the same sex in the same column with different superscripts differ significantly, ($P<0.05$)

Table 2.5 Serum clinical chemistry parameters of Wistar rats fed diets containing soybean trypsin inhibitors and/or DL-ethionine

Diet	Cholesterol mg/dL	Triglycerides mg/dL	Urea Nitrogen mg/dL	Total protein g/dL	SGPT IU/mL	Alkaline phosphatase IU/L
a) Male rats						
SPI control	60 ± 10^{a}	227 ± 30^{a}	6.9 ± 0.3^{ab}	4.3 ± 0.3	19 ± 4^{a}	385 ± 40^{a}
Casein control	74 ± 9^{ab}	281 ± 27^{a}	10.4 ± 2.1^{b}	3.8 ± 0.7	18 ± 2^{a}	489 ± 97^{a}
Basal	89 ± 7^{b}	316 ± 52^{ab}	15.4 ± 0.8^{c}	4.0 ± 0.4	26 ± 3^{ab}	662 ± 98^{ab}
Basal + DL-ETH	92 ± 12^{b}	458 ± 34^{b}	19.1 ± 1.0^{d}	3.9 ± 0.3	68 ± 9^{c}	1162 ± 97^{c}
Basal + DL-MET	71 ± 3^{ab}	243 ± 15^{a}	5.8 ± 0.4^{a}	3.5 ± 0.3	20 ± 2^{ab}	394 ± 54^{a}
Basal + DL-ETH + DL-MET	76 ± 7^{ab}	215 ± 38^{a}	6.0 ± 0.8^{a}	4.2 ± 0.3	24 ± 2^{ab}	439 ± 64^{a}
SPI control + DL-ETH	82 ± 12^{ab}	437 ± 90^{b}	9.9 ± 1.5^{b}	4.0 ± 0.2	33 ± 4^{b}	808 ± 157^{b}
b) Female rats						
SPI control	74 ± 14	124 ± 17^{a}	11.5 ± 1.0^{ab}	4.1 ± 0.3	20 ± 4^{a}	257 ± 36^{a}
Casein control	62 ± 13	197 ± 46^{ab}	16.4 ± 1.0^{cd}	4.5 ± 0.3	22 ± 1^{a}	446 ± 93^{a}
Basal	64 ± 9	238 ± 39^{ab}	18.3 ± 0.6^{d}	3.4 ± 0.4	33 ± 3^{a}	523 ± 92^{a}
Basal + DL-ETH	104 ± 22	757 ± 105^{c}	17.5 ± 2.5^{cd}	3.4 ± 0.3	124 ± 6^{b}	1745 ± 198^{c}
Basal + DL-MET	52 ± 11	120 ± 11^{a}	8.5 ± 1.0^{a}	4.1 ± 0.1	28 ± 2^{a}	243 ± 30^{a}
Basal + DL-ETH + DL-MET	62 ± 9	204 ± 55^{ab}	10.5 ± 0.9^{ab}	4.1 ± 0.2	29 ± 6^{a}	386 ± 75^{a}
SPI control + DL-ETH	63 ± 21	362 ± 54^{b}	13.9 ± 1.7^{bc}	4.0 ± 0.4	26 ± 1^{a}	948 ± 121^{b}

Means ± SEM for 6 to 8 animals per group.
Means of values for rats of the same sex in the same column with different superscripts differ significantly, ($P<0.05$).

Table 2.6 Serum-free essential amino acids* in female Wistar rats fed diets containing soybean trypsin inhibitors and/or DL-ethionine

Diet	Arg	His	Leu	Ile	Met	Phe	Thr	Trp	Val	Lys	Total Essential
SPI control	30.9^{e}	7.2^{a}	15.9^{a}	11 2^{c}	8.1^{a}	6.5^{ab}	68^{ab}	8.6^{c}	16.6^{c}	91^{d}	271^{b}
Casein control	16.6^{c}	5.4^{a}	12.1^{a}	8 1^{abc}	7.5^{a}	5.7^{a}	82^{ab}	7.0^{bc}	17.1^{c}	61^{b}	230^{ab}
Basal	24.7^{de}	9.3^{a}	15.2^{a}	10.1^{bc}	4.1^{a}	9.0^{bc}	105^{bc}	6.0^{ab}	15.7^{c}	83^{cd}	291^{b}
Basal + DL-ETH	0^{a}	29.2^{b}	61.7^{b}	5.9^{a}	7.0^{a}	10.7^{c}	178^{c}	4.1^{a}	15.2^{c}	32^{a}	360^{c}
Basal + DL-MET	25.1^{de}	6 9^{a}	10.0^{a}	6 9^{ab}	9.9^{a}	6.9^{ab}	32^{ab}	5.2^{ab}	9.4^{ab}	53^{b}	173^{a}
Basal + DL-ETH + DL-MET	19.6^{cd}	7.2^{a}	52.3^{b}	8.6^{abc}	28.6^{c}	5.7^{a}	22^{a}	5.2^{ab}	7.9^{a}	65^{bc}	228^{ab}
SPI control + DL-ETH	7.9^{b}	8.0^{a}	66.4^{b}	8.7^{abc}	16.4^{b}	7.8^{abc}	46^{ab}	5.4^{ab}	14.0^{bc}	60^{b}	252^{ab}
SEM**	2.0	1.4	5.2	0 9	1.7	0.8	22.4	0.6	1.4	5.5	23

* μM/100 mL serum.

** SEM calculated from analysis of variance data.
Means with different superscripts in the same column differ significantly ($P<0.05$).

Table 2.7 Serum-free non-essential amino acids* in female Wistar rats fed diets containing soybean trypsin inhibitors and/or DL-ethionine

Diet	Gly	Pro	Ser	Orn	Tau	∝-Aba	Met SO
SPI control	42.9^{c}	20.0^{ab}	48.8^{a}	21.1^{ab}	17.8^{bc}	0.8^{b}	0^{a}
Casein control	19 6^{a}	30.3^{c}	30.9^{a}	12.5^{a}	13.4^{b}	0 2^{a}	0^{a}
Basal	35.3^{bc}	23.4^{abc}	94.9^{b}	27.0^{bc}	4.8^{a}	0^{a}	0^{a}
Basal + DL-ETH	114.6^{e}	24 8^{bc}	187.3^{c}	93.3^{d}	25.0^{d}	1.4^{c}	45 6^{d}
Basal + DL-MET	26.4^{ab}	16 8^{a}	26.4^{a}	14.8^{a}	23.2^{cd}	1.1^{bc}	3 4^{a}
Basal + DL-ETH + DL-MET	23.9^{ab}	19.3^{ab}	23.3^{a}	14.2^{a}	20.6^{cd}	1.2^{bc}	21.6^{b}
SPI control + DL-ETH	58 9^{d}	26.2^{bc}	42.7^{a}	36.4^{c}	19.1^{bcd}	1.2^{bc}	35.8^{c}
SEM**	4.4	2.0	12.3	3.1	1.9	0.1	4.5

* μM/100 mL serum.

** SEM calculated from analysis of variance data.
Means with different superscripts in the same column differ significantly ($P<0.05$).

3. INFLUENCE OF DIETARY SOYBEAN TRYPSIN INHIBITORS AND DL-ETHIONINE ON SULFUR AMINO ACID ADEQUACY OF DIETS FOR YOUNG RATS

INTRODUCTION

The reported enhancement of chemically-induced pancreatic cancers and the development of nodular hyperplasia and adenocarcinomas in rats fed soy products containing trypsin inhibitors (Gumbmann et al. 1986, McGuinness et al. 1981) has led to concern over the potential influence of soybean trypsin inhibitors (SBTI) on human health (Roebuck 1987, Wormsley 1988). In humans, substantial dietary intakes of trypsin inhibitors may occur in vegetarians and in infants fed only soy-based products. The use of soy protein isolates for enhancing function or protein quality of processed foods and the increasing consumption of traditional Japanese soy products in North America (Wang 1984) indicate other potential sources of SBTI in the human diet.

There has also been interest in the influence of deficits in dietary lipotropes (choline, vitamin B_{12}, folic acid and sulfur amino acids) on methionine metabolism, the hypomethylation of DNA and the development of cancer (Hoffman 1984, Rogers and Newberne 1980). Inadequate dietary methionine also leads to reduced growth and accumulation of other essential amino acids in the blood of growing rats (Peace et al. 1986). Ethionine the carcinogenic analogue of methionine, competes with methionine in the transmethylation-transsulfuration pathway, inhibiting methyl transfer reactions and may result in hypomethylation of DNA (Shivapurkar et al. 1984).

Soybean trypsin inhibitors irreversibly bind sulfur amino acid-rich pancreatic proteases in the gut into inactive complexes which are lost via the feces. In rats, this results in increased protease production and pancreatic hypertrophy and hyperplasia (Green and Lyman 1972, Gumbmann et al. 1986). The net loss of sulfur amino acids may increase their requirements in growing animals and a deficit of them may be a factor in the development of pancreatic cancer reported to occur in long term studies of rats fed soy-based diets.

This study was carried out to see if changes in growth, clinical chemistry and serum amino acid parameters of young rats fed SBTI, in diets theoretically adequate for rat growth, were indicative of a prolonged deficit in the lipotrope methionine. Because of the influence of the hepatocarcinogen DL-ethionine on methionine metabolism, it was of interest to see if the effects of dietary SBTI and DL-ethionine were similar or synergistic. This finding could point to the potential mechanisms for the effects of SBTI.

MATERIALS AND METHODS

Diets containing 20% protein from ANRC Casein (Humko Sheffield Chemical, Division of Kraft Inc., Madison, WI) or from either of two combinations of soy protein isolate (Supro 710, Ralston Purina Co., St. Louis, MO) and ground raw soybeans (Maple Arrow Soybeans, Hardy Seeds Ltd., Inkerman, Ont.) provided three levels of SBTI (0, 448 or 808 mg trypsin inhibited per 100 g diet) as determined by the method of Smith et al. (1980). Provision of equivalent dietary protein level was ensured by assay of crude protein content (% N x 6.25) (in duplicate) for each protein source by means of a micro-Kjeldahl technique using a Kjeltec 1030 Auto Analyzer (Tecator Instruments, Hoganas, Sweden) and the use of results obtained for diet formulation. DL-ethionine (Sigma Chemical Co., St. Louis, MO) at three levels (0.

0.05 and 0.10% of diet), was combined with the three different levels of SBTI to give a total of 9 test diets. Supplementary L-methionine, L-threonine and L-glutamic acid (Sigma Chemical Co.) were added to meet rat growth requirements (National Research Council 1978) and to make each diet isonitrogenous and matching in essential amino acids. Other dietary components are shown in Table 3.1. Diets were prepared monthly and stored in a cold room at 5°C.

Weanling male Wistar rats (Charles River Canada Inc., St. Constant, Que.) were randomly assigned to one of the 9 test diets on the basis of matched initial body weights in a randomized complete block design involving 24 replicates. Rats were housed in individual stainless steel mesh-bottomed cages and maintained at 22°C with 12-h light-dark cycles Food and water were provided ad libitum and food intakes and weight gains were recorded weekly. At the end of 4, 8 and 12 weeks, rats in 8 replicates were placed under halothane anaesthesia. The liver of each rat was exposed by a mid ventral incision and a small portion of the central lobe was clamped off using liquid nitrogen-chilled tongs. This liver clamp sample was weighed and extracted as described in Section 5 for analysis of S-adenosyl derivatives. Each anesthetized rat was subsequently exsanguinated using a plastic syringe and 19 gauge needle, via the abdominal aorta. Serum was separated by centrifugation for 10 min at 1600 x g in an IEC CENTRA-7R centrifuge (International Equipment Co., Damon Corp., Needham Heights, MA) and aliquots were deproteinized with acetonitrile (1.1, vol.vol) and stored with undeproteinized serum at -70°C. The remaining liver tissue, pancreas and kidneys were quickly removed from each animal, weighed, frozen in liquid nitrogen and stored at -70°C until thawed for analysis.

Serum clinical chemistry values (serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), urea nitrogen, triglycerides and cholesterol) for

individual animals were determined in duplicate, using an ABA-200 series II Bichromatic Autoanalyzer (Abbott Diagnostics Division, Dallas, TX) and Abbott Clinical Chemistry test kits (Abbott Diagnostics Division, Mississauga, Ont.).

Pairs of thawed, deproteinized serum samples from rats on the same diet at the same time period, were pooled and analyzed for free amino acids by a modification of the Waters Pico Tag method for protein hydrolysates involving phenylisothiocyanate derivatization and reverse phase HPLC (Sarwar and Botting 1990) using a Waters HPLC system and a Waters Pico Tag Amino Acid column (Waters Scientific, Mississauga, Ont.).

Two different statistical analyses were made. The first involved separate analyses of variance for a randomized complete block design at 4, 8 and 12 weeks, in each case using the 9 diets as treatments. Differences between treatment means were determined by Duncan's multiple range test (Snedecor and Cochran 1967, Duncan 1955). The second analysis examined the effects of dietary levels of SBTI, DL-ethionine as well as weeks on test (time) as a 3 x 3 x 3 factorial design. The least significant difference test was used to determine differences between treatment means (Snedecor and Cochran 1967). Both analyses employed the PCANOVA program (Human System Dynamics, Northridge, CA).

RESULTS

The effects on growth parameters of young male rats fed diets containing increasing levels of SBTI with and without DL-ethionine are shown in Tables 3.2, 3.3, 3.4, and 3.5. Statistical analysis for these tables was by the first method described. Increased dietary SBTI without ethionine caused lower weight gains (Table 3.2) and feed efficiencies (Table 3.4) over the 12 weeks of the experiment. Feeding ethionine without SBTI also resulted in decreases

relative to rats fed the control diet without SBTI or ethionine although differences were not significant at 12 weeks. Combination of dietary SBTI and ethionine at highest levels tested resulted in poorest animal growth performance with up to 37, 35 and 27% lower weight gains and 18, 18 and 19% lower feed efficiencies compared to control animals (0 SBTI, 0 ethionine) after 4, 8 and 12 weeks respectively.

Table 3.4B lists F-ratios for the above growth parameters, obtained by a separate analysis of variance for a three factorial design using levels of dietary SBTI and ethionine and weeks on test (time) as factors. The significant interaction of SBTI level x time for total weight gain (Table 3.4B) involved greater increases in total weight gain at 8 and 12 weeks at 0 compared to intermediate or high levels of SBTI. Increasing dietary ethionine also caused decreased total weight gains when averaged over all times and levels of dietary SBTI

Feed efficiency varied at different times in response to dietary ethionine. At 4 and 8 weeks, increased dietary ethionine caused lower feed efficiencies whereas at 12 weeks no effect was observed. Presence of dietary SBTI when averaged over all ethionine levels and times also decreased feed efficiency.

Absolute liver weight (not shown) decreased in response to increasing levels of SBTI in the diet at 4, 8 and 12 weeks, although a similar trend was not significant when weights were expressed relative to body weights (Table 3.5). Increasing dietary ethionine resulted in higher relative liver weights compared to animals fed the control diet, and this could be seen at each level of SBTI fed. The interaction of dietary ethionine level with time for relative liver weight detected by the factorial analysis (Table 3.5B), involved significantly lower relative liver weights at each level of ethionine tested as time on test increased, although weights decreased in response in increasing ethionine at each week measured. Addition of SBTI to

the diets, when averaged over all ethionine levels and times, caused a drop in relative liver weight although this was significant only at the intermediate level of SBTI tested.

Absolute pancreatic weight (not shown) and pancreatic weight relative to body weight (Table 3.5) were higher in rats fed increased SBTI without ethionine although increases at 8 and 12 weeks were not significant. Relative pancreatic weights did not change with increased dietary ethionine alone although lowest relative pancreatic weight occurred in rats fed SBTI combined with the highest level of ethionine. The factorial analysis of relative pancreatic weight revealed a significant interaction of SBTI and ethionine (Table 3.5B). Relative pancreatic weight averaged over all times, increased with increasing SBTI at the 0 level of dietary ethionine. At 0.05% ethionine however, no significant change occurred with increasing dietary SBTI and at 0.10% ethionine relative pancreatic weight decreased with increasing SBTI.

Relative kidney weights (Table 3.5) were higher with increased dietary SBTI alone at 4 and 12 weeks, but not with increased dietary ethionine alone. The combination of highest SBTI and ethionine resulted in lower relative kidney weight at 4 weeks compared to animals fed high SBTI alone. A significant three way interaction of SBTI x ethionine x time was indicated for relative kidney weight by factorial analysis (Table 3.5B). Examination of the SBTI x ethionine interaction indicated that increased SBTI caused higher weights only at the 0 ethionine level. When the SBTI x time interaction was examined in the absence of ethionine, relative kidney weight was consistently higher at 4 weeks compared to 8 or 12 weeks for each level of SBTI fed.

Serum total cholesterol was lower in rats fed increased dietary SBTI without ethionine (Table 3.6) and this pattern continued throughout the study. Cholesterol also was lower, compared to controls in rats fed ethionine without SBTI or combination of SBTI and ethionine.

Factorial analysis of serum cholesterol (Table 3.6B) revealed significant interactions of dietary SBTI x ethionine and ethionine x time. Averaged over all time periods, serum cholesterol levels decreased with addition of SBTI in rats fed diets without ethionine but underwent no further decrease beyond the low and constant level caused by 0.05% and 0.10% ethionine. Averaged over all SBTI levels, the response of serum cholesterol to dietary ethionine also varied with weeks on test. At 0% ethionine, cholesterol levels were constant over the 12 weeks, but consistently higher than at 0.05% or 0.10% ethionine. At 8 and 12 weeks equivalent low cholesterol levels occurred for both 0.05 and 0.10% ethionine compared to rats fed diets without ethionine, whereas at 4 weeks, 0.10% ethionine resulted in cholesterol levels intermediate to those of rats fed either 0 or 0 05% ethionine.

Serum triglyceride levels, in contrast to cholesterol, increased in response to dietary ethionine at 4 weeks and were highest in rats fed SBTI and ethionine. Triglyceride levels generally appeared to decrease as the rats aged with lowest levels measured after 12 weeks (Table 3.6). Significant interactions of SBTI x ethionine, SBTI x time, and of ethionine x time were detected for serum triglycerides by the factorial analysis (Table 3 6B). Averaged over all weeks, with 0 or 0.05% dietary ethionine, triglycerides showed no change with increasing SBTI and remained at equivalent low levels. At 0.10% ethionine, serum triglycerides were higher than in rats fed 0 or 0.05% ethionine and underwent a marked increase in response to highest level of dietary SBTI. Averaged over all ethionine levels, triglycerides at 4 weeks were consistently higher than at 8 or 12 weeks and increased in response to increased dietary SBTI. The lower triglyceride concentrations in rats at 8 and 12 weeks showed no response to increasing SBTI. Similarly, averaged over all levels of SBTI, serum triglycerides were higher at 4 weeks than at 8 or 12 weeks and increased in response to increasing ethionine At 8 and

12 weeks, lower triglyceride levels did not change significantly with increased dietary ethionine.

Presence of SBTI without ethionine resulted in lower serum urea nitrogen (Table 3.6) at each time measured. No significant change in urea nitrogen occurred in response to dietary ethionine without SBTI whereas rats fed combination of SBTI and ethionine had increased urea nitrogen compared to rats fed SBTI alone. Significant main effects of SBTI, ethionine and time were detected for urea nitrogen by the factorial analysis (Table 3.6B). Dietary SBTI caused increased urea nitrogen, increasing ethionine caused a decrease and levels were higher at 8 weeks compared to 4 weeks.

Rats fed diets containing SBTI without ethionine had higher serum glutamate pyruvate transaminase (SGPT) (Table 3.7) than controls at 4 and 8 weeks, as did rats fed dietary ethionine without SBTI. Increases at 12 weeks were not significant. The combination of SBTI and ethionine also resulted in increased SGPT relative to the controls with highest levels at 4 and 8 weeks measured in rats fed the highest combination of SBTI and ethionine. The significant interaction of ethionine x time (Table 3.7B) involved a significant increase in SGPT in response to increasing ethionine at 4 weeks that was not seen at 8 or 12 weeks. When averaged over all weeks and ethionine levels, SGPT also increased with presence of dietary SBTI as also demonstrated by the first statistical analysis.

Serum glutamate oxaloacetate transaminase (SGOT) activity showed no significant change with increased dietary SBTI without ethionine and decreased only in response to 0.05% dietary ethionine without SBTI at 4 weeks and in response to both 0.05% and 0.10% ethionine when the intermediate level or SBTI was fed (Table 3.7). Factorial analysis revealed significant main effects of ethionine and time (Table 3.7B). Increased dietary ethionine

averaged over all SBTI levels and times, resulted in decreased SGOT. SGOT also decreased with increasing weeks on test when averaged over all levels of SBTI and ethionine.

Free amino acids in serum were measured at 4, 8 and 12 weeks. Table 3.8 shows concentrations of selected amino acids in serum after 4 and 12 weeks of feeding the various diets. At 4 weeks, increased dietary SBTI and ethionine both resulted in lower serum methionine concentrations. Using the factorial analysis, a significant 3 way interaction of SBTI x ethionine x time was detected for serum free methionine (Table 3.9B). Examination of the interaction of SBTI x ethionine indicated highest methionine in control rats (0 SBTI, 0 % ethionine) and decreases with increasing SBTI in the absence of ethionine and with ethionine in the absence of SBTI. At 0.05% and 0.10% dietary ethionine, lower serum free methionine showed no further significant decrease in response to increased SBTI. Looking at the interaction of SBTI x time at the 0 ethionine level, it was found that the greatest methionine decrease in response to SBTI occurred at 4 weeks where both intermediate and high levels of dietary SBTI resulted in lower serum methionine compared to control rats. At 8 weeks, at 0% dietary ethionine, serum methionine was significantly lower only at the highest SBTI level and at 12 weeks levels did not change in response to SBTI.

Serum levels of several other essential amino acids also decreased with increasing dietary SBTI. However, relatively greater increases in serum threonine (Table 3.8) and arginine (not shown), resulted in an overall accumulation of total essential amino acids. Increased dietary ethionine without SBTI had no significant effect on serum threonine, and dietary combination of SBTI and ethionine did not appear to effect levels compared to those of rats fed SBTI alone. The factorial analysis (Table 3.9B) however, indicated significant interactions of SBTI x ethionine as well as of SBTI x time for serum free threonine. When averaged over all time periods, threonine was consistently elevated with higher SBTI and

remained constant with increased ethionine except for a decrease with intermediate level of SBTI and 0.05% ethionine compared to rats fed intermediate SBTI and 0 or 0.10% ethionine. When averaged over all ethionine levels, highest concentrations and greatest increases of threonine in response to increasing dietary SBTI occurred at 4 weeks compared to 8 or 12 weeks. At 12 weeks, threonine levels and increases in response to increased SBTI were less than at 8 weeks.

Serum concentrations of total free essential amino acids (Table 3.8) increased in rats fed higher levels of SBTI but showed no significant change with increasing ethionine at 4 or 12 weeks. Combination of dietary SBTI and ethionine maintained the higher levels of total essential amino acids seen in rats fed increased SBTI alone. After 12 weeks of feeding, essential amino acid concentrations in serum tended to be lower in all rats, although a similar pattern of higher levels of total essential amino acids and threonine in response to dietary SBTI alone and in combination with ethionine was maintained. These findings were generally confirmed by the factorial analysis (Table 3.9b). Averaged over all ethionine levels, the highest serum concentrations and greatest increases in total essential amino acids in response to increasing SBTI occurred at 4 weeks and declined at 8 and 12 weeks. When averaged over all SBTI levels, total essential amino acids were consistently higher at 4 weeks at each level of dietary ethionine and decreased with increasing weeks on test. At 0.05 % and 0.10% ethionine decreases between week 8 and 12 were not significant. When averaged over all time periods, total essential amino acids increased to equivalent and highest concentrations at highest dietary SBTI with each level of ethionine fed. However, the pattern of increase varied. Total essential amino acids increased at both intermediate and high levels of dietary SBTI with 0 and 0.10% ethionine but increased only in response to the highest level of SBTI with 0.05% dietary ethionine.

Serum concentrations of non essential amino acids related to the transsulfuration pathway of methionine metabolism also changed relative to levels in control rats, in response to SBTI and/or DL-ethionine (Table 3.9).

Serum free serine increased with increasing dietary SBTI at 4 and 12 weeks (Table 3.9). It also increased at the highest level of ethionine without SBTI, although this was significant only at 4 weeks. Rats fed combinations of SBTI and ethionine had serum serine concentrations equivalent or lower than those of rats fed the same level of SBTI without ethionine although levels were always higher than in control rats. Serine concentrations decreased over time as confirmed by the factorial analysis. Significant interactions of SBTI x time, ethionine x time and SBTI x ethionine were demonstrated (Table 3.9B). Averaged over all ethionine levels, serine concentrations and increases with increasing dietary SBTI, seen at each week, were greatest at 4 weeks. Averaged over all SBTI levels, serum serine decreased to equivalent levels over 12 weeks at each level of ethionine fed but significant decreases occurred more gradually at the 0 level of ethionine than at the highest ethionine level. When averaged over all times, serine increase with increased dietary SBTI was greatest at the 0 compared to the 0.05% or 0.10% ethionine levels.

Elevated serum glycine compared to levels in control rats, occurred with feeding of both SBTI and ethionine alone at 4 and 12 weeks. Highest 4-week concentrations were measured in rats fed diets containing both SBTI and ethionine (Table 3 9). A significant second order interaction of SBTI x ethionine x time was detected (Table 3.9B). Examination of the interaction of ethionine x time indicated that the rise in serum glycine with increasing ethionine was most pronounced at 4 weeks. The SBTI x ethionine interaction at 4 weeks also demonstrated lowest serum glycine in control rats with greatest proportional increases

occurring in response to higher SBTI in the absence of ethionine. Highest glycine levels however, occurred in rats fed 0.10% ethionine with increased SBTI.

No consistent changes were seen in serum alanine at 4 or 12 weeks although highest 4-week serum concentrations occurred in rats fed higher levels of SBTI and ethionine (Table 3.9). Factorial analysis indicated a significant second order interaction of SBTI x ethionine x time (Table 3.9B). Examination of the SBTI x time interaction revealed that when averaged over all ethionine levels, alanine changed (increased) only at 4 weeks. The SBTI x ethionine interaction at 4 weeks, showed that alanine increased with increasing SBTI only with 0.05% and 0.10% dietary ethionine.

Serum free taurine decreased in response to dietary SBTI at 4 weeks but not at 12 weeks, and increased in response to dietary ethionine alone at both 4 and 12 weeks (Table 3.9). Combination of high dietary SBTI and ethionine usually moderated the high serum taurine seen in rats fed ethionine alone. Significant interactions of SBTI x time and of ethionine x time were detected for taurine (Table 3.9B). When averaged over all dietary ethionine levels, taurine showed greatest decrease at 4 weeks with increasing dietary SBTI. Lower levels at 8 and 12 weeks underwent no further decrease with increased SBTI. Averaged over all SBTI levels, taurine increased with increasing dietary ethionine at 4 and 12 weeks but not at 8 weeks, with greatest increases occurring at 4 weeks.

DISCUSSION

The level of dietary lipotropes provided by the various diets used in the current study were theoretically adequate for the growing rat (National Research Council 1978). Hence any induction of lipotrope deficiency and, more specifically, sulfur amino acid deficiency should

have resulted from the influence of SBTI and/or DL-ethionine ingestion. Younger rats with higher requirements for essential amino acids for growth would be particularly susceptible to such deficiency.

Changes in blood amino acids have also long been used to determine animal amino acid requirements and to identify limiting amino acids in protein sources (Sarwar et al. 1983, Young et al. 1972). Plasma or serum concentrations of essential amino acids can indicate adequacy of dietary methionine relative to animal growth requirements. Low serum methionine, elevated levels of other essential amino acids and reduced growth and protein synthesis occur with insufficient methionine. Achievement of methionine adequacy relative to growth requirements is characterized by decreases in serum levels of essential amino acids and of serine (transsulfuration substrate), and glycine and alanine (precursors to serine) (Keith et al. 1972, Peace et al. 1986).

The decrease in serum methionine with increasing dietary SBTI without ethionine, in the growing rats of the current study, likely reflected decreased methionine availability or its increased utilization and requirement due to the effects of SBTI on protein digestibility and on synthesis and loss of sulfur amino acid-rich pancreatic proteases (Rackis and Gumbmann 1981). The lack of further decreases in methionine with increased dietary SBTI in the presence of ethionine suggested that the effects of two dietary factors on methionine were not additive, though both the antilipotrope ethionine and SBTI decreased serum free methionine.

The accumulation of the essential amino acid threonine and of total essential amino acids with increasing SBTI further suggested methionine deficit and decreased protein synthesis. As for methionine, responses were greatest at 4 weeks, likely reflecting the higher methionine requirements and hence greater sensitivity of the younger animals.

The accumulation of serum free levels of the transsulfuration substrate serine in response to SBTI suggested a negative influence of SBTI on the transsulfuration pathway. The supply of homocysteine co-substrate, derived via transmethylation and transsulfuration, may have been limited due to SBTI effects on methionine availability and supply.

A transsulfuration deficit was further supported by increases in the serine precursor glycine, which was at highest concentration at 4 weeks in rats fed SBTI and ethionine combined, and accumulated with increasing SBTI and ethionine at 4 and 12 weeks. The second order interaction of SBTI x ethionine x time, confirmed that as for other amino acid changes, changes to glycine were most pronounced at 4 weeks, again reflecting the greater sensitivity of the younger animals to induced methionine deficits. The second order interaction also indicated that SBTI had the greatest influence on increasing serum glycine although the responses of both were additive since highest levels at 4 weeks occurred in rats fed combinations of SBTI and highest ethionine.

The increase in the serine precursor alanine, at 4 weeks with increasing SBTI required the presence of dietary ethionine. Highest levels occurred with combination of highest dietary levels of SBTI and ethionine. Accumulation of alanine as for glycine may have served to moderate changes in serum serine in response to dietary ethionine.

The decrease in serum levels of the transsulfuration end-product taurine, with increased dietary SBTI, also seen at 4 weeks, further supported the idea of a deficit in the transsulfuration pathway in response to SBTI although increased fecal excretion of bile acids conjugated with taurine may have occurred in response to higher intakes of soy protein and plant fibre with increased intake of soy-derived SBTI. It is suggested that the accumulation of serum free taurine in response to increased ethionine was not due to increased taurine production via transsulfuration, but rather resulted from decreased taurine utilization in bile

acid conjugation due to the toxic effects of ethionine on liver lipid metabolism and transport. Significant decreases in circulating cholesterol could serve to decrease utilization of taurine.

Weight gain and nitrogen retention are traditional methods for determining sulfur amino acid adequacy of diets (Rao et al.1959, Stockland et al. 1973).

Growth deficits as manifested by decreases in weight gain, relative liver weight and feed efficiency, that occurred with increasing dietary SBTI and/or ethionine also suggested inadequacy or unavailability of dietary sulfur amino acids. The fact that the lowest total weight gains and feed efficiencies were observed with combination of dietary SBTI and ethionine suggests that SBTI may serve to exacerbate the negative growth effects of ethionine (or vice versa) (Table 3.2).

The greater increase in total weight gain in the absence of SBTI at 8 and 12 weeks compared to increases with 0.05% or 0.10% ethionine reflect the ongoing influence of SBTI over the 12 weeks of the test. The antilipotrope ethionine also caused decreased weight gain and this was detected by both statistical analyses.

The significant decreases in feed efficiency at 4 and 8 weeks and the lack of change at 12 weeks in response to increased dietary ethionine suggested a greater capacity of the older animals to resist the negative growth effects of ethionine likely mediated by decreased methionine requirements or improved ethionine detoxification mechanisms. As also demonstrated by the initial analysis, the significant main effect of SBTI involved a decrease in feed efficiency as dietary SBTI increased. Such decreases likely resulted from decreased protein digestibility, amino acid availability and other negative influences of dietary SBTI (Rackis and Gumbmann 1981).

Higher liver weight relative to body weight of rats fed ethionine may have resulted from increased hepatic lipid deposition. Averaged over all SBTI levels, highest relative liver weights

occurred at 4 weeks but decreased over time at each level of dietary ethionine. This likely reflected a combination of decreased relative liver weights with increasing maturation of the animals combined with continued hepatic lipid deposition due to the toxic and antilipotropic effects of ethionine. Farber (1967) previously reported that acute injections of ethionine into rats caused rapid liver lipid accumulation. The combination of SBTI and ethionine showed no significant interaction although the lowering of relative liver weight with increased dietary SBTI averaged over all times and ethionine levels, suggested a protective effect of SBTI against lipid deposition. The development of fatty liver is a major feature of most lipotrope deficits, likely caused by abnormalities in hepatic lipid secretion and deposition (Farber 1967, Pascale et al. 1982).

Increased relative pancreatic weight measured at 4 weeks in rats fed increasing levels of SBTI without ethionine (Table 3.5) were indicative of the known pancreatic hypertrophic and hyperplastic effects of SBTI (Rackis and Gumbmann 1981). The absence of such a response at 8 and 12 weeks may have reflected the decreased susceptibility of older animals to SBTI perhaps in part mediated by decreased sulfur amino acid requirements for growth (Nitsan and Liener 1976a). The moderation of SBTI-induced pancreatic weight increase in the presence of ethionine likely resulted from a decreased capacity of the animals for pancreatic trophic response to SBTI. The competition or inhibition of ethionine and derivatives in the transmethylation-transsulfuration pathway of methionine metabolism (Alix 1982) would tend to decrease the supply of cysteine required for enhanced protease synthesis and release (Barnes and Kwong 1965, Liener 1976).

Based upon the three way interaction of SBTI x ethionine x time for relative kidney weight (Table 3.5B), dietary SBTI in the absence of ethionine, either induced kidney hypertrophy or retarded the maturation or growth of the animal, resulting in higher kidney

weight relative to body weight. Based upon the demonstrated effects of increased dietary SBTI on body weight gains and feed efficiency, the latter seems more likely. At the 0 ethionine level, the SBTI x time interaction for relative kidney weight, involving significantly higher weights at 4 weeks versus 8 or 12 weeks for each level of SBTI, likely reflected time- or maturation-induced changes in relative kidney weight.

Dietary SBTI and the antilipotrope ethionine both caused decreased serum cholesterol suggesting similar antilipotropic effects. However, ethionine appeared to be the most hypocholesterolemic and the influence of the two dietary factors did not appear to be additive. The decrease in cholesterol with dietary ethionine continued over the 12 weeks of the study suggesting ongoing lipotrope deficit. Based upon the first statistical analysis, the decrease in cholesterol with increasing SBTI without ethionine was also prolonged.

The lower cholesterol and the trend towards lower serum triglyceride levels in response to dietary SBTI without ethionine suggested that SBTI may contribute to the hypocholesterolemic effects of dietary soy protein. The hypolipidemic (hypocholesterolemic) effects of dietary soy proteins in several species are well known and have been proposed to result from changes in cholesterol metabolism, including absorption, synthesis, deposition and secretion (Carroll 1983, Hamilton and Carroll 1976). It is suggested that SBTI may play a role in such changes possibly through induced changes in absorption and secretion.

The increase in serum triglycerides with high dietary ethionine alone or with combinations of dietary SBTI and ethionine, contrasted to decreases in serum cholesterol. This may have been a toxic effect of the antilipotrope, since acute ethionine injections have been reported to cause rapid increase in liver triglyceride levels (Farber 1967). The observation that serum triglycerides decreased at 8 and 12 weeks suggests an adaptive response of rats to this dietary insult.

A high negative correlation was shown between plasma urea nitrogen levels and biological value (Eggum 1970). The lower urea nitrogen levels in rats fed dietary SBTI with and without ethionine contrasted to an earlier report of a rise in serum urea nitrogen in response to ingestion of raw soy or increased dietary SBTI (Sitren et al. 1985). In the current study, attempts were made to balance dietary fibre by inclusion of Alphacel (at 5% diet for the casein-based control diet and at lower levels in the soy-based diets to account for fibre levels in the raw soy). The fermentable fibre of the raw soy, in contrast to the relatively inert Alphacel, would tend to stimulate bacterial activity in the lower intestine, enhancing production of short chain fatty acids, lowering production and mucosal reabsorption of ammonia and decreasing serum urea levels (Vince et al. 1990). In addition, decreased digestibility of raw soy-containing diets combined with the formation and excretion of inactivated protease-inhibitor complexes via the feces could further have contributed to losses of dietary nitrogen resulting in lower serum urea nitrogen levels.

Dietary protein inadequacy was further suggested by elevations in SGPT in response to SBTI. An inverse relationship between protein quality and SGPT was previously suggested (Bodwell 1975). Based on the amino acid changes already discussed, such protein inadequacy may have been mainly caused by a sulfur amino acid deficit. The highest level of SGPT, measured in rats fed the combination of SBTI and 0.1% ethionine at 4 weeks, was likely a toxic response that appeared to moderate in older rats. SGPT and SGOT are normally intracellular enzymes that undergo serum increases in response to liver damage. The response of SGPT, in the case of liver damage, has been reported to be more pronounced than that of SGOT (Tietz 1983).

The findings of the current study would suggest that even with theoretical adequacy of dietary lipotropes, it is possible to induce a sulfur amino acid deficit by means of feeding

SBTI to young rats. Such a deficit is manifested by reduced growth and changes in levels of serum amino acids related to methionine metabolism as well as by changes in certain clinical chemistry parameters. It is evident that the methionine analogue ethionine can also serve to modify these parameters and that in some instances combination of dietary ethionine and SBTI can exacerbate these changes. The continuing deficits in animal growth parameters and prolonged changes in serum clinical chemistry parameters indicated that lipotrope deficiency as induced by SBTI and/or DL-ethionine can have an ongoing influence in the growing rat. This is further supported by changes in concentrations of serum free amino acids related to dietary methionine adequacy and the transsulfuration pathway. Moderation of changes in serum free amino acids and other parameters over time suggest a reduced methionine requirement of the older rats.

TABLE 3.1 Composition of diets

Ingredient	Percent of Diet
Protein (Casein[1], Soy protein isolate[2]/soybeans[3]	20.0
L-Met, L-Thr, L-Glu[4]	+
DL-Eth[5]	±
AIN-76 Mineral mix[6]	3.5
AIN-76A Vitamin mix[6]	1.0
Alphacel[7]	5.0
Choline bitartrate	0.2
Soybean oil	15.0
Corn starch	~55.3

[1] Animal Nutrition Research Council Casein (Humko Sheffield, Kraft Inc., Norwich, NY).
[2] Supro 710 (Ralston Purina Co, St. Louis, MO).
[3] Maple Arrow Soybeans, raw, ground (Hardy Seeds Ltd, Inkerman, Ont.).
[4] Added in amounts making diets isonitrogenous and matching in essential amino acids (Sigma Chemical Co, St. Louis, MO).
[5] Added at 0, 0.05 or 0.10% of diet (Sigma Chemical Co., St. Louis, MO).
[6] American Institute of Nutrition (ICN Biomedicals, Cleveland, OH).
[7] Amount of added fibre varied to compensate for fibre content of raw seeds.
[8] Amount of added oil varied to compensate for oil of raw seeds.
[9] Canada Starch Co., Toronto, Ont.

TABLE 3.2 Effects of feeding SBTI and DL-ethionine on weight gains[1,2] of young male rats

DL-ethionine (%)	0			0.05			0.10			SEM[4]
SBTI[3]	0	448	808	0	448	808	0	448	808	
				Weight gain (g)						
4 weeks	193±14^{f}	181±11^{ef}	174±16de	170±16de	162±14^{cd}	149±13^{bc}	162±15^{cd}	137±17^{b}	122±13^{a}	5
8 weeks	398±33^{f}	354±16de	340±39^{cde}	364±21^{e}	321±27^{bcd}	312±28^{bc}	335±47^{cde}	299±20^{b}	260±18^{a}	11
12 weeks	481±43^{d}	415±53^{bc}	427±49^{bc}	455±28^{cd}	414±46^{bc}	396±56^{ab}	460±45^{cd}	359±26^{a}	363±24^{a}	15

[1]Values are means ± SD for 8 rats per diet.
[2]Means with different superscripts in the same row differ significantly, ($P < 0.05$).
[3]SBTI. soybean trypsin inhibitor measured as mg trypsin inhibited per 100 g diet.
[4]SEM calculated from analysis of variance data.

TABLE 3.3 Effects of feeding SBTI and DL-ethionine on feed intake[1,2] of young male rats

DL-ethionine (%)	0			0.05			0.10			SEM[4]
SBTI[3]	0	448	808	0	448	808	0	448	808	
				Food intake (g)						
4 weeks	406±24^{e}	403±18^{e}	395±28de	389±27^{cde}	392±21^{cde}	366±39^{bc}	370±24^{bcd}	343±34^{b}	312±28^{a}	9
8 weeks	1035±69^{e}	996±58de	984±75^{cde}	947±53^{bcd}	920±51^{bc}	921±60^{bc}	932±84^{bcd}	881±47^{b}	795±58^{a}	22
12 weeks	1594±115^{b}	1522±113^{ab}	1586±136^{b}	1538±36^{ab}	1489±99^{ab}	1441±160^{a}	1517±94^{ab}	1415±78^{a}	1443±73^{a}	39

[1]Values are means ± SD for 8 rats per diet.
[2]Means with different superscripts in the same row differ significantly, ($P < 0.05$).
[3]SBTI: soybean trypsin inhibitor measured as mg trypsin inhibited per 100 g diet.
[4]SEM calculated from analysis of variance data.

TABLE 3 4 Effects of feeding SBTI and DL-ethionine on feed efficiency[1,2] of young male rats

DL-ethionine (%)	0			0.05			0.10			SEM[4]
SBTI[3]	0	448	808	0	448	808	0	448	808	
	(g wt. gain/100 g intake)									
4 weeks	47.5±2.2^{c}	44.9±1.8^{b}	44.0±1.9^{b}	43.7±1.8^{b}	41.3±2.1^{a}	41.0±2.5^{a}	43.9±2.1^{b}	39.9±2.8^{a}	39.0±1.8^{a}	0.78
8 weeks	40.5±3.6^{c}	35.5±1.0^{b}	34.3±1.7^{ab}	38.6±2.9^{c}	34.9±1.4^{ab}	33.7±1.3^{ab}	35.9±2.1^{b}	33.9±1.7^{ab}	32.7±1.1^{a}	0.74
12 weeks	31.0±1.8^{d}	27.2±1.9^{c}	26.9±0.8^{bc}	29.6±1.5^{d}	26.8±1.4^{bc}	27.5±1.8^{c}	30.3±1.6^{d}	25.4±1.4^{ab}	25.1±1.5^{a}	0.55

[1]Values are means ± SD for 8 rats per diet.
[2]Means with different superscripts in the same row differ significantly, ($P < 0.05$).
[3]SBTI: soybean trypsin inhibitor measured as mg trypsin inhibited per 100 g diet.
[4]SEM calculated from analysis of variance data.

TABLE 3.4.B. F-ratios for growth parameters of young rats fed different levels of soybean trypsin inhibitors and DL-ethionine over 12 weeks.

Factor	F-ratios[1]		
	Total weight gain	Total feed intake	Feed Efficiency
SBTI	58.70**	1.73	90.07**
% DL-Ethionine (E)	50.53**	2.46	37.88**
Weeks on (T)	1292.06**	0.88	1129.02**
SBTI X E	1.81	49.14	0.73
SBTI X T	5.67**	1.04	0.99
E X T	1.02	1.18	6.58**
SBTI X E X T	0.75	1.00	1.59
SEM[2]	10.94	244.40	0.37

[1] ** $P<0.01$, * $P<0.05$.

[2] SEM: standard error of mean calculated from analysis of variance with 189 degrees of freedom.

TABLE 3 5 Effects of feeding SBTI and DL-ethionine on organ weights[1,2] of young male rats

DL-ethionine (%)		0			0.05			0.10			SEM[4]
SBTI[3]		0	448	808	0	448	808	0	448	808	
	Weeks										
Liver weight (g/100 g body wt.)	4	5 03± 38^{ab}	4 82±.18^{a}	4.84± 28^{a}	5 72± 48^{cd}	5.28± 34^{abc}	5.36±.40^{bc}	5 56± 49^{cd}	5 94±.62^{d}	5.63±.58^{cd}	0.15
	8	3.94± 22^{ab}	3.73± 20^{a}	3 76± 24^{a}	4 25± 21^{bc}	3.96±.17^{ab}	4.30±.29^{bc}	4.38± 44^{c}	3.94± 55^{ab}	4.29±.40^{bc}	0.12
	12	3 24± 30^{abc}	3 08± 16^{a}	3 14± 23^{ab}	3 62± 27^{cde}	3.37± 31^{bcd}	3 47±.37^{cd}	3.80±.23^{e}	3 66±.28de	3.61± 13de	0 10
Pancreas Weight (g/100 g body wt.)	4	0 56±.10^{ab}	0.61±.19^{b}	0.80±.11^{c}	0.57± 08^{ab}	0.54±.12^{ab}	0.47±.13^{a}	0.56±.07^{ab}	0.47±.07^{a}	0.48±.16^{a}	0.04
	8	0 59± 08^{cd}	0 61± 11^{cd}	0.67±.05^{d}	0.57±.11^{c}	0 52± 10^{bc}	0.51±.06^{abc}	0 58±.11^{cd}	0.46± 06^{ab}	0 42± 11^{a}	0.03
	12	0 63±.06^{b}	0 62±.08^{b}	0 68± 11^{b}	0 60±.09^{b}	0 62±.11^{b}	0.58±.13^{b}	0.59±.08^{b}	0 47±.06^{a}	0 48±.05^{a}	0.03
Kidney weight (g/100 g body wt.)	4	0 87± 03^{a}	0.92± 06^{a}	1.01± 15^{b}	0 89±.06^{a}	0 88±.10^{a}	0.91±.06^{a}	0 96±.11^{ab}	0 89± 07^{a}	0.90±.06^{a}	0.03
	8	0 67± 05^{a}	0 70± 06^{abc}	0 69± 05^{abc}	0 68± 04^{ab}	0 74± 04^{c}	0 72± 04^{bc}	0 68± 04^{ab}	0 70± 05^{abc}	0 72±.05^{abc}	0.02
	12	0 50± 07^{ab}	0 60± 04^{cd}	0 64±.04^{d}	0 57± 06^{bc}	0 49±.13^{a}	0 53± 07^{cd}	0 55±.03^{abc}	0 61± 05^{cd}	0 58± 04^{cd}	0 02

[1]Values are means ± SD for 8 rats per diet
[2]Means with different superscripts in the same row differ significantly ($P < 0.05$)
[3]SBTI. Soybean trypsin inhibitor measured as mg trypsin inhibited per 100 g diet.
[4]SEM calculated from analysis of variance data.

TABLE 3.5.B. F-ratios for organ weights (g/100 g body weight) of young rats fed different levels of soybean trypsin inhibitor and DL-ethionine over 12 weeks.

Factor	F-ratios[1]		
	Liver weight	Pancreas weight	Kidney weight
SBTI	5.73**	2.00	7.21**
% DL-Ethionine (E)	53.31**	35.06**	1.28
Weeks on (T)	562.25**	2.50	473.71**
SBTI X E	0.93	9.61**	3.78**
SBTI X T	0.28	0.61	1.24
E X T	2.45**	1.13	1.98
SBTI X E X T	1.41	1.22	3.10**
SEM[2]	0.12	0.04	0.03

[1] ** $P<0.01$, * $P<0.05$.

[2] SEM: standard error of mean calculated from analysis of variance with 189 degrees of freedom.

TABLE 3.6 Effects of feeding SBTI and DL-ethionine on serum clinical parameters[1,2] of young male rats

DL-ethionine (%)		0			0.05			0.10			SEM[4]
SBTI[3]		0	448	808	0	448	808	0	448	808	
	Weeks										
Cholesterol (mg/dL)	4	98±15^{c}	77±9^{b}	73±9^{b}	55±6^{a}	56±13^{a}	57±6^{a}	68±10^{ab}	60±8^{a}	74±24^{b}	4
	8	105±11^{c}	76±11^{b}	74±6^{b}	59±12^{a}	52±8^{a}	53±7^{a}	58±7^{a}	57±13^{a}	51±5^{a}	3
	12	96±11^{c}	70±7^{c}	74±14^{b}	56±15^{a}	50±6^{a}	47±3^{a}	50±9^{a}	47±10^{a}	53±7^{a}	3
Triglycerides (mg/dL)	4	257±40^{a}	299±40^{ab}	308±44^{ab}	285±55^{ab}	327±49^{b}	326±59^{b}	431±96^{c}	450±59^{c}	586±98^{d}	21
	8	211±39^{a}	184±32^{a}	195±51^{a}	219±52^{a}	175±45^{a}	199±47^{a}	183±48^{a}	192±57^{a}	314±78^{b}	17
	12	191±26^{abc}	149±31^{a}	157±26^{ab}	196±38^{c}	153±35^{a}	160±42^{abc}	173±30^{abc}	182±28^{abc}	186±41^{abc}	12
Urea nitrogen (mg/dL)	4	23±3^{cd}	15±2^{a}	15±3^{a}	21±2^{cd}	18±3^{bc}	16±3^{ab}	25±5^{d}	22±3^{cd}	20±3^{cd}	1
	8	24±3^{d}	19±2^{ab}	20±3^{abc}	23±4^{cd}	18±2^{a}	20±3^{abc}	24±5^{d}	21±4^{bcd}	22±2^{cd}	1
	12	22±2^{bc}	17±3^{a}	16±2^{a}	20±3^{b}	18±2^{a}	20±1^{b}	24±2^{c}	21±3^{bc}	21±3^{bc}	1

Values are means ± SD for 8 rats per diet

[2]Means with different superscripts in the same row differ significantly ($P < 0.05$)

[3]SBTI Soybean trypsin inhibitor measured as mg trypsin inhibited per 100 g diet

[4]SEM calculated from analysis of variance data

TABLE 3.6.8. F-ratios for serum clinical parameters of young rats fed different levels of soybean trypsin inhibitor and DL-ethionine over 12 weeks.

Factor	F-ratios[1]		
	Cholesterol	Triglycerides	Urea Nitrogen
SBTI	23.50**	10.48**	40.33**
% DL-Ethionine (E)	150.88**	56.50**	22.51**
Weeks on (T)	10.99**	284.42**	5.51**
SBTI X E	12.19**	8.67**	2.38
SBTI X T	1.12	6.60**	1.74
E X T	4.18**	28.71**	2.33
SBTI X E X T	1.03	1.63	0.62
SEM[2]	3.81	18.08	1.06

[1] ** $P<0.01$, * $P<0.05$.

[2] SEM: standard error of mean calculated from analysis of variance with 189 degrees of freedom.

TABLE 3.7 Effects of feeding SBTI and DL-ethionine on serum enzymes[1,2] of young male rats

DL-ethionine (%)		0			0.05			0.10			SEM[6]
SBTI[3]		0	448	808	0	448	808	0	448	808	
	Weeks										
SGPT[4] (IU/L)	4	24±3^{a}	30±7^{ab}	32±6^{b}	28±5^{ab}	32±6^{b}	30±3^{ab}	35±7^{bc}	41±11^{c}	42±10^{c}	2
	8	20±4^{a}	29±4^{b}	28±3^{b}	30±8^{bc}	28±8^{b}	29±8^{b}	26±4^{b}	25±6^{ab}	35±6^{c}	2
	12	23±4	26±5	30±7	30±9	30±6	35±12	30±5	30±2	29±4	2
SGOT[5] (IU/L)	4	113±27^{c}	107±25^{bc}	98±23^{abc}	92±11^{ab}	98±27^{abc}	82±17^{a}	102±19^{bc}	92±19^{ab}	90±26^{ab}	6
	8	74±12^{bcd}	77±14^{d}	75±8^{bcd}	76±8^{cd}	66±8^{ab}	67±10^{abc}	66±7^{ab}	62±9^{a}	70±10^{abcd}	3
	12	87±13^{b}	86±15^{b}	79±11^{ab}	75±9^{ab}	76±9^{ab}	85±20^{b}	78±15^{ab}	75±11^{ab}	67±6^{a}	4

[1]Values are means ± SD for 8 animals
[2]Means with different superscripts in the same row differ significantly ($P < 0.05$)
[3]SBTI: Soybean trypsin inhibitor measured as mg trypsin inhibited per 100 g diet.
[4]SGPT: Serum glutamate pyruvate transaminase
[5]SGOT Serum glutamate oxaloacetate transaminase.
[6]SEM calculated from analysis of variance data.

TABLE 3.7.B. F-ratios for serum clinical chemistry parameters of young rats fed different levels of soybean trypsin inhibitors and DL-ethionine over 12 weeks.

Factor	F-ratios[1]	
	SGPT	SGOT
SBTI	11.62**	2.09
% DL-ethionine (E)	12.58**	9.12**
Weeks on (T)	10.50**	53.48**
SBTI X E	1.86	0.32
SBTI X T	0.92	1.08
E X T	5.44**	1.00
SBTI X E X T	1.62	1.07
SEM[2]	2.30	5.63

[1] ** $P<0.01$, * $P<0.05$.

[2] SEM: standard error of mean calculated from analysis of variance with 189 degrees of freedom.

TABLE 3.8 Effects of feeding SBTI[1] and DL-ethionine on serum free essential amino acids[2,3] in young rats

DL-ethionine (%)		0			0.05			0.10			
SBTI[1]		0	448	808	0	448	808	0	448	808	SEM[4]
	Weeks										
L-Met	4	10.8^{d}	8.5^{bc}	8.1^{ab}	9.2^{c}	8.6^{bc}	7.6^{ab}	8.0^{ab}	7.2^{a}	7.2^{a}	0.3
	12	8.1^{bcd}	7.7^{abc}	7.7^{abc}	6.7^{a}	6.9^{ab}	7.7^{abc}	9.2^{d}	7.8^{abc}	8.8^{cd}	0.4
L-Thr	4	102.7^{a}	219.1^{bc}	282.4^{de}	74.3^{a}	166.2^{b}	312.9^{e}	78.4^{a}	213.2^{bc}	239.3^{cd}	19.0
	12	56.6^{a}	83.9^{bc}	100.5^{de}	49.8^{a}	76.2^{b}	110.4^{e}	53.5^{a}	91.1^{cd}	98.1^{de}	4.4
Total essential amino acids	4	309.7^{ab}	389.5^{cd}	447.8^{de}	272.6^{ab}	388.7^{bc}	488.0^{e}	255.7^{a}	369.9^{cd}	416.8^{d}	22.5
	12	217.5^{a}	237.0^{ab}	254.1^{bc}	224.3^{a}	231.7^{ab}	261.0^{bc}	254.3^{bc}	259.1^{bc}	274.6^{c}	9.1

[1]SBTI: Soybean trypsin inhibitor measured as mg trypsin inhibited per 100 g diet.
[2]μmoles/100 mL serum, means ± SEM of 4 pooled samples (2 rats per pool).
[3]Means with different superscripts in the same row differ significantly, ($P < 0.05$).
[4]SEM calculated from analysis of variance data.

TABLE 3.9 Effects of feeding SBTI[1] and DL-ethionine on serum free non-essential amino acids[2,3] in young rats

DL-ethionine (%)		0			0.05			0.10			
SBTI[1]		0	448	808	0	448	808	0	448	808	SEM[4]
	Weeks										
L-Ser	4	37.0^{a}	52.0^{c}	57.3^{d}	36.3^{a}	43.7^{b}	48.0^{c}	42.1^{b}	50 4^{c}	51.2^{c}	1.4
	12	33.6^{a}	40.0^{bcd}	43.0^{e}	34.5^{a}	37.9^{bc}	42.2^{de}	36.7^{ab}	40.4^{cde}	43.8^{e}	1.1
L-Gly	4	28.5^{a}	54.1^{c}	51.9^{bc}	47.2^{b}	72.4^{d}	71.0^{d}	69.8^{d}	102.9^{e}	102.8^{e}	2.0
	12	26.6^{a}	45.8^{b}	42.4^{b}	46.5^{b}	67.4^{d}	72.9^{ef}	55.4^{c}	76.8^{f}	68.8^{de}	1.7
L-Ala	4	71.6^{ab}	65.4^{a}	70.6^{ab}	64.0^{a}	64.6^{a}	79.5^{cd}	66.9^{a}	76.2^{bc}	85.9^{d}	2.3
	12	72.8^{d}	64.0^{bc}	65.1^{bc}	60.8^{bc}	50.9^{a}	63.0^{bc}	65.7^{bc}	58.7^{b}	66.9^{cd}	2.3
L-Tau	4	25.1^{c}	20.3^{b}	15.8^{a}	24.7^{c}	24.2^{c}	16.9^{a}	31.5^{d}	29.8^{d}	26.1^{c}	0.8
	12	18.4^{a}	20.1^{ab}	19.3^{ab}	20 7^{ab}	23.2^{cd}	23.1^{cd}	24.9^{d}	25.2^{d}	22.1^{bc}	0.7

[1]SBTI: Soybean trypsin inhibitor measured as mg trypsin inhibited per 100 g diet.
[2]μmoles/100 mL serum, means ± SEM of 4 pooled samples (2 rats per pool).
[3]Means with different superscripts in the same row differ significantly, ($P < 0.05$).
[4]SEM calculated from analysis of variance data.

TABLE 3.9.B F-ratios for serum free amino acid parameters of young rats fed different levels of soybean trypsin inhibitors and DL-ethionine over 12 weeks.

Factor	F-ratio[1]						
	L-Met	L-Thr	Total essential amino acids	L-Ser	L-Gly	L-Ala	L-Tau
SBTI	13.60**	181.77**	70.31**	68.61**	290.18**	19.34**	14.97**
% DL-ethionine (E)	7.07**	5 35**	2.35	15.03**	558 37**	17.17**	37.16**
Weeks on (T)	6.61**	180.37**	136 88**	42.53**	50.07**	30.82**	19.40**
SBTI X E	4.36**	3.88**	2 61*	4 49**	1.20	10.92**	0.79
SBTI X T	6.84**	28.63**	16 10**	5.08**	3.18*	7.47**	9.15**
E X T	14.73**	1 49	3.06*	3.61**	23.14**	8.55**	9.46**
SBTI X E X T	4 48**	1.64	1 85	1 55	2.68*	2 58*	2.01
SEM[2]	0 33	12 38	16 25	1.74	2.31	2.52	1 26

[1] ** P<0.01, * P<0 05

[2] SEM standard error of mean calculated from analysis of variance with 81 degrees of freedom.

4. EFFECTS OF SULFUR AMINO ACID SUPPLEMENTATION ON GROWTH, CLINICAL CHEMISTRY AND SERUM AMINO ACID PARAMETERS OF RATS FED SOYBEAN TRYPSIN INHIBITORS AND DL-ETHIONINE

INTRODUCTION

The influence of deficits of dietary lipotropes (methionine, choline, vitamin B_{12} and folate) on animal growth and metabolism and on the development of various forms of cancer in the presence or absence of chemical carcinogens has been amply demonstrated (Newberne et al. 1983, Ghoshal and Farber 1984, Yokoyama 1985). Dietary soybean trypsin inhibitors (SBTI) have similarly been shown to influence animal growth performance and to contribute to pancreatic carcinogenesis in rats (Rackis and Gumbmann 1981, McGuinness et al. 1984).

The mechanism of lipotrope-influenced carcinogenesis is unknown although in terms of hepatocarcinogenesis, Ghoshal et al. (1983) proposed that continual hepatic necrosis, lipid accumulation and resultant hepatocyte proliferation were involved. Altered methionine metabolism, hypomethylation of DNA and abnormal gene expression in proliferating cells have also been implicated (Hoffman 1984). The mechanisms involved in SBTI-influenced carcinogenesis are also unknown although pancreatic cell damage and proliferation occur in response to dietary SBTI (Morgan et al. 1984, Roebuck 1986).

Boyd et al. (1985) demonstrated growth deficits, elevated markers of hepatic injury and lipid accumulation in rats fed a lipotrope deficient diet. It was previously shown (Sections III.2 and III.3) that many of these symptoms occurred in rats fed SBTI. Decreased availability or utilization of the lipotrope methionine in response to dietary SBTI was further suggested

by changes in serum free amino acids related to the transsulfuration pathway of methionine metabolism. Combination of SBTI with the carcinogenic methionine analogue ethionine, increased many of these symptoms of lipotrope deficit.

The effectiveness of supplementary sulfur amino acids in overcoming the negative growth and pancreatic trophic effects of naturally occurring inhibitors of soy and other legumes has been much studied. There are conflicting reports concerning the relative efficacy of cystine compared to methionine supplements and the mechanisms involved in response to cystine (Kwong and Barnes 1963, Frost and Mann 1966, Finkelstein et al. 1982b, Liener and Kakade 1980).

Cystine is sparing of methionine requirements for growth, although this capacity appears to be influenced by both the level of dietary protein and the ratio of cystine to methionine (Byington et al. 1972, James and Hove 1980). In raw soy-based diets providing low, intermediate and high levels of dietary protein, Gumbmann and Friedman (1987) reported that supplementary cystine enhanced rat growth performance, although not as effectively as methionine. They also found that supplements of cysteine and cysteine derivatives increased relative pancreatic weights of rats fed raw soy-based diets.

The carcinogenic effects of ethionine involve in part a negative influence on methionine metabolism (Farber 1967, Brada et al. 1976). The requirements, availability and/or utilization of sulfur amino acids are also involved in the negative growth and pancreatic trophic effects of SBTI (Rackis and Gumbmann 1981).

It was earlier demonstrated that dietary SBTI induced symptoms of lipotrope deficit similar to those induced by DL-ethionine, in young male rats. Soybean trypsin inhibitors also appeared to exacerbate many of the lipotropic effects of ethionine. It was decided to examine the influence of increasing amounts of SBTI and a subcarcinogenic dose of ethionine on

development of a lipotrope deficit in young rats fed diets meeting rat growth requirements. Because of the intimate involvement of sulfur amino acids in the effects of both dietary factors it was decided to examine the potential protective effects of sulfur amino acid supplementation on lipotrope deficits caused by intake of SBTI combined with ethionine.

MATERIALS AND METHODS

Diets providing 20% protein from either of two combinations of soy protein isolate (Supro 710, Ralston Purina Co., St. Louis, MO) and ground raw soybeans (Maple Arrow Soybeans, Hardy Seeds Ltd., Inkerman, Ont.) provided two levels of soybean trypsin inhibitors (421 and 775 mg trypsin inhibited per 100 g diet) as determined by the method of Smith et. al. (1980). Supplementary L-methionine, L-threonine, and L-tryptophan (Sigma Chemical Co., St. Louis, MO) were added to ensure that the diets met rat growth requirements (National Research Council 1978) and matched the amino acid composition of a 20% protein diet based on ANRC Casein (Humko Sheffield Chemical Division, Kraft Inc., Madison, WI) which was also formulated. Additional diets based upon the soy protein isolate/raw soybean diets were also prepared with addition of 0.2% DL-ethionine with and wihout supplementary L-methionine or L-cysteine (3.35mmol/100g diet). Appropriate amounts of L-glutamic acid were added to the diets to make them isonitrogenous. Other dietary components are listed in Table 4.1.

Weanling male Wistar rats (Charles River Canada Inc., St. Constant, Que.) were randomly assigned on the basis of matched initial body weights to one of the 9 diets in a randomized complete block design involving 16 replicates. Rats were housed in individual stainless steel mesh-bottomed cages and maintained at 22°C with 12-h light-dark cycles. Food and tap water were provided ad libitum and food intakes and weight gains were

determined weekly. At the end of 2 and 4 weeks, animals in the same 8 replicates were placed under halothane anaesthesia, and exsanguinated via the abdominal aorta subsequent to obtaining a small amount of liver tissue via a midventral incision and a liver clamp technique that employed liquid N_2-chilled tongs. The freeze-clamped liver tissues were for use in other studies (Section III-5). Serum was separated by centrifugation for 10 min at 1600 x g in an IEC CENTRA-7R centrifuge (International Equipment Co., Damon Corp., Needham Heights, MA) and aliquots were deproteinized with acetonitrile (1:1, vol:vol). Deproteinized extracts and remaining undeproteinized serum were stored at -70°C. Remaining liver and pancreas, spleen and kidney tissues were quickly removed from each animal, weighed, frozen in liquid nitrogen and stored at -70°C until thawed for analysis. Prior to freezing, portions of liver, pancreas and kidney tissues from 4 rats on each diet, at each time period, were removed and fixed in 10% buffered formalin for histopathological examination.

Serum clinical chemistry values (cholesterol, triglycerides, urea nitrogen, glutamate pyruvate transaminase (SGPT) and glutamate oxaloacetate transaminase (SGOT) for individual animals were determined in duplicate, using an ABA-200 series II Bichromatic Autoanalyzer (Abbott Diagnostics Division, Dallas, TX) and Abbott Clinical Chemistry test kits (Abbott Diagnostics Division, Mississauga, Ont.). Quality control was assured by using freshly diluted human Moni-Trol.ES Level 1 Chemistry Control samples (American Dade, American Hospital Supply Corp., Miami, FL) in each run of the autoanalyzer.

Pairs of thawed, deproteinized serum samples from rats on the same diet and time period were pooled and analyzed for serum free amino acids by a modification of the Waters Pico Tag method for protein hydrolysates involving phenylisothiocyanate derivatization and reverse phase HPLC (Sarwar and Botting 1990) using a Waters HPLC system and a Waters Pico Tag Amino Acid column (Waters Scientific, Mississauga, Ont.). Chromatograms of

elution profiles of a set of PTC-amino acid standards (Figure 4.1) and of an actual rat serum PTC-amino acid preparation (Figure 4.2) are shown.

Analysis of variance was made using the PCANOVA program (Human System Dynamics, Northbridge CA) and significant differences between means were determined by Duncan's Multiple Range Test (Duncan 1955). Results are reported as means ± SEM.

RESULTS

Weekly growth parameters of the rats fed the different diets are shown in Table 4.2. Weight gains, food intakes and food efficiencies of rats fed the casein-based diet (Diet 1) and the diets containing the two different levels of SBTI (Diets 2 and 6) were equivalent at each time measured, except during the second week when food efficiency of rats fed the highest level of SBTI was slightly lower.

Addition of 0.2% DL-ethionine at the two levels of dietary SBTI (Diets 3 and 7) resulted in marked weight gain deficits and decreased food intakes relative to rats fed the same levels of SBTI without ethionine. Food efficiency was also lower in rats fed the lowest level of SBTI with ethionine during the first, second and fourth weeks and in rats fed the highest level of SBTI with ethionine during the first and fourth weeks.

Growth improved with methionine supplementation of SBTI + ethionine-containing diets (Diets 4 and 8) although weight gains equivalent to those of rats fed SBTI without ethionine were only achieved in rats fed the lower level of SBTI with ethionine and supplemental methionine after three weeks. Feed intake also increased with methionine supplementation of ethionine-containing diets at both levels of SBTI but not to the levels seen in rats fed diets with the same levels of SBTI without ethionine. Deficits in feed efficiency

resulting from combination of SBTI and ethionine were prevented by presence of supplementary methionine.

L-cysteine supplementation (Diets 5 and 9) was ineffective at preventing the weight losses and decreased food intake occurring in rats fed the two combinations of SBTI and ethionine. Food efficiency was also frequently lower in rats receiving cysteine compared to methionine supplementation.

Organ weights of rats sacrificed at 2 and 4 weeks are shown in Tables 4.3 and 4.4 respectively. Both absolute liver weight and liver weight per 100g body weight (relative liver weight) of rats fed the casein-based diet or the two SBTI-containing diets were equivalent. Presence of ethionine resulted in lower absolute liver weights at both 2 and 4 weeks in rats fed the two different levels of SBTI. Relative liver weights of rats fed SBTI + ethionine-containing diets did not differ from controls except for rats fed the highest level of SBTI with ethionine. At 2 weeks their relative liver weights were significantly greater. Methionine supplementation prevented lower absolute liver weight measured at 2 and 4 weeks in rats fed both levels of SBTI with added ethionine. This supplementation also resulted in higher relative liver weight compared to rats fed the SBTI + ethionine diets although this was significant only at 4 weeks. Absolute liver weights of rats fed cysteine-supplemented, SBTI + ethionine-containing diets were similar to those of rats fed SBTI + ethionine diets without supplementary sulfur amino acids and lower than those of rats receiving methionine supplements. Cysteine supplementation did not result in the increase in relative liver weight seen at four weeks with methionine supplementation of the SBTI + ethionine containing diets.

Absolute and relative pancreatic weight increased in response to increasing dietary SBTI without ethionine at both 2 and 4 weeks. Addition of ethionine to SBTI-containing diets caused a decrease in absolute and relative pancreatic weight. In the case of relative

pancreatic weight, the decrease was significant only at 4 weeks. Methionine supplementation did not appear to affect relative pancreatic weight but moderated the decrease in absolute pancreatic weight occurring in rats fed the ethionine-containing diets. At 4 weeks, absolute pancreatic weights of rats fed ethionine + SBTI diets with supplementary methionine were lower than those of rats fed SBTI diets without ethionine. Cysteine supplementation did not prevent the lower absolute pancreatic weights at 2 and 4 weeks or the lower relative pancreatic weights at 4 weeks occurring in rats fed SBTI combined with ethionine.

Absolute and relative kidney weights of the animals fed the casein-based and two soy-based diets were equivalent at both 2 and 4 weeks, although at 4 weeks, no significant differences occurred in relative kidney weights of animals on any of the diets. Ethionine addition resulted in a significant drop in absolute kidney weights at both 2 and 4 weeks. Relative kidney weight increased in animals fed ethionine at lowest level of dietary SBTI at 2 weeks but showed no significant change at the higher SBTI level at 2 weeks or at either dietary SBTI level at 4 weeks. Supplementary methionine increased absolute kidney weights over those of rats fed the SBTI + ethionine diets but not to the level of rats fed the SBTI diets without ethionine with the exception of rats fed the lower level of SBTI with added ethionine and methionine at 4 weeks. At 2 and 4 weeks, relative kidney weights of rats fed supplementary methionine did not differ from those of rats receiving the SBTI-containing diets with ethionine. Rats fed supplementary cysteine had the same low absolute kidney weights of rats fed the SBTI-containing diets with ethionine although relative kidney weights did not differ from those of rats fed SBTI-containing diets with and without ethionine or with ethionine + methionine.

Rats fed the two levels of SBTI also had the same absolute spleen weights which at 4 weeks, were equivalent to those of the casein-fed rats. At 2 weeks, absolute spleen

weights were higher in casein-fed rats than in rats fed the lower level of SBTI without ethionine. Relative spleen weights of rats on these three diets were similar at both 2 and 4 weeks. Ethionine addition resulted in a sharp drop in absolute spleen weights. Relative spleen weights, although lower did not differ significantly from those of animals fed the SBTI-containing diets without ethionine. Methionine supplementation partially prevented the drop in absolute spleen weights occurring in rats fed the SBTI + ethionine-containing diets, whereas cysteine supplementation did not. Relative spleen weights were frequently higher with methionine supplementation of SBTI + ethionine-containing diets but not with cysteine supplementation.

Histopathological assessment of hepatic, renal and pancreatic tissues at 5, 20 and 50x magnification revealed no significant lesions diagnostic of disease processes in rats from any of the diet groups. Livers in many cases exhibited evidence of mitoses although no consistent pattern with respect to diet groups was observed. Pancreatic lesions, which involved a few instances of zonal fatty infiltration, occurred in rats fed supplementary sulfur amino acids at both levels of SBTI and ethionine tested.

The most noticeable abnormality observed was a gradation of renal calcinosis in rats fed diets containing SBTI and ethionine. This was not detected in rats fed diets in which ethionine was lacking. Most frequent and severe calcinosis occurred in rats fed the highest level of SBTI and ethionine followed by rats fed the lower level of SBTI and ethionine. Kidneys of rats fed supplementary cysteine showed a moderate degree of calcinosis especially at the higher level of dietary SBTI. Methionine supplementation resulted in a minimal degree of calcinosis with most occurring in rats fed the higher level of SBTI.

Serum clinical chemistry parameters of rats fed the different diets over 2 and 4 weeks are shown in Tables 4.5 and 4.6 respectively. At both time periods, serum cholesterol and

serum triglyceride levels were equivalent in casein-fed rats (Diet 1) and in rats fed the two soy-based diets with increasing levels of SBTI (Diets 2 and 6). Combination of SBTI and DL-ethionine (Diets 3 and 7) resulted in a marked increase in serum triglycerides at both 2 and 4 weeks. Higher serum cholesterol was also measured although this was not significant for rats fed the lower level of SBTI (Diet 3). Methionine supplementation of SBTI + ethionine-containing diets resulted in normalization of serum triglycerides and lower serum cholesterol. Rats consuming cysteine-supplemented diets retained the high levels of triglycerides and cholesterol measured in rats fed SBTI + ethionine diets.

Serum urea nitrogen concentrations were similar in rats fed each or the three diets without ethionine at 2 weeks, but decreased with increasing dietary levels of SBTI without ethionine at 4 weeks. Higher urea nitrogen levels occurred when ethionine was added to the SBTI-containing diets although this was not significant at 4 weeks in rats fed the lower level of SBTI. At 2 weeks, methionine supplementation of the ethionine-containing diets at both levels of SBTI used, resulted in lowering of urea nitrogen to concentrations similar to those of rats fed at the same level of dietary SBTI without ethionine. At 2 weeks, cysteine supplementation did not result in lower serum cholesterol. Differences in urea nitrogen levels at 4 weeks with added methionine or cysteine to the SBTI + ethionine-containing diets were less pronounced.

Concentrations of SGPT and SGOT were not affected by increasing dietary SBTI without ethionine. Addition of ethionine increased SGPT and SGOT, although at 4 weeks, higher SGPT in rats fed the highest level of trypsin inhibitor with ethionine did not differ significantly from the level in rats fed the corresponding diet without ethionine. Methionine supplementation normalized SGPT and SGOT levels at both time periods measured, while equimolar cysteine supplementation did not.

Serum free essential amino acid concentrations at 2 and 4 weeks are shown in Tables 4.7 and 4.8 respectively. Serum essential amino acid concentrations were equivalent in rats fed the two soy-based diets with increasing levels of SBTI but frequently differed from concentrations in casein-fed rats.

Rats fed the two SBTI-containing diets with added ethionine had much lower serum arginine and threonine at 2 and 4 weeks and lower lysine at 2 weeks compared to rats fed the SBTI diets without ethionine. Lower serum leucine and valine were also frequently measured, especially at the higher level of dietary SBTI. Serum phenylalanine and methionine in contrast, were higher compared to rats fed SBTI diets without ethionine.

Serum levels of many essential amino acids changed in response to feeding methionine-supplemented, SBTI + ethionine diets. At 2 weeks, isoleucine, leucine, threonine, tryptophan and valine were lower at both levels of SBTI. Phenylalanine was also lower in rats fed the higher level of SBTI with added ethionine and methionine. At 4 weeks, isoleucine and phenylalanine were lower in rats fed both levels of SBTI with ethionine and added methionine. Valine was also lower in rats fed the lower level of SBTI with added ethionine and threonine lower in rats fed the higher level of SBTI with ethionine and methionine.

Increases in serum concentrations of essential amino acids also occurred in rats fed methionine-supplemented diets. At 2 weeks, arginine and lysine were higher in rats fed the lower level of SBTI + ethionine and, arginine, histidine, methionine and lysine higher at the higher level of SBTI + ethionine when supplementary methionine was included. At 4 weeks, arginine and methionine, and arginine, histidine and methionine were higher in the rats fed the low and high levels of SBTI with added ethionine and methionine. Serum concentrations of several essential amino acids were frequently lower in rats fed methionine-supplemented SBTI + ethionine containing diets compared to rats fed the same level of SBTI without ethionine.

Cysteine supplemented rats generally retained serum free essential amino acid concentrations of rats fed SBTI + ethionine diets, showing fewer changes compared to those receiving methionine supplementation. At 2 weeks, arginine was higher and tryptophan was lower at the lowest level of dietary SBTI, and histidine and leucine were higher at the highest level of SBTI. At 4 weeks, arginine was lower at the lower level or dietary SBTI and phenylalanine and tryptophan were lower at the higher dietary SBTI level.

Serum levels of free non-essential amino acids and derivatives are shown in Tables 4.9 and 4.10. As for essential amino acids, levels of individual free non-essential amino acids in rats fed the soy-based diets providing increasing amounts of SBTI were generally similar, although some differences were measured. Of interest are non-essential amino acids related to sulfur amino acid metabolism.

Serum serine increased with increasing dietary SBTI without ethionine (Diets 1, 2 and 6). Alanine and glycine were consistently higher and taurine lower in rats fed the higher compared to the lower level of SBTI without ethionine (Diet 2 compared to Diet 6) at both 2 and 4 weeks although differences were not significant. The level of α-aminobutyric acid was significantly lower at 2 weeks at both levels of dietary SBTI and at the lower level of dietary SBTI at 4 weeks compared to rats fed the casein-based diet without SBTI (Diet 1).

The presence of dietary ethionine resulted in a significant drop in serum serine at the highest dietary level of SBTI but appeared not to have an effect at the lower SBTI level. Serum alanine and glycine increased at 2 and 4 weeks at both levels of dietary SBTI although the increase in alanine was not significant at 2 weeks at the higher level of SBTI. Serum levels of taurine and α-aminobutyrate also increased in ethionine treated rats compared to those fed SBTI without ethionine.

Supplementary methionine resulted in lower serine, alanine, glycine, and α-

aminobutyrate but higher taurine compared to rats fed the SBTI + ethionine containing diets. Supplementary cysteine was generally not effective in lowering levels of these amino acids from the higher levels in rats fed the corresponding SBTI + ethionine containing diets.

Serum ornithine also increased markedly in response to combination of dietary SBTI and ethionine and this increase was moderated by supplementary methionine but not by cysteine.

Totals of serum free essential amino acids, non-essential amino acids and related parameters at 2 and 4 weeks (Tables 4.11 and 4.12 respectively) reflected the findings for individual amino acids already described. Total essential amino acids in rats fed the two different levels of SBTI (Diets 2 and 6) were equivalent to each other and to levels in casein-fed rats (Diet 1) except for rats fed the lower level of SBTI at 4 weeks when total essential amino acids were lower than those in casein-fed rats. At 2 weeks, total non-essential amino acids were equivalent in sera of rats fed the casein-based diet and the soy-based diet with lower SBTI contents, and higher in the high SBTI soy-based diets. At 4 weeks, levels in all three diets were equivalent. A similar pattern was seen for total amino acids and for ratios of essential to total amino acids.

The addition of ethionine at both levels of dietary SBTI resulted in lower total essential amino acids, higher total non-essential amino acids and higher total amino acids at both times measured. The ratios of essential to total amino acids decreased with ethionine addition to both SBTI-containing diets at 2 and 4 weeks.

Methionine supplementation of the two SBTI + ethionine diets resulted in lower levels of total essential amino acids at both 2 and 4 weeks, although differences were not significant at the higher level of SBTI intake. Rats fed the SBTI + ethionine diets with supplemental methionine also had lower levels of total essential amino acids than did rats fed the two SBTI

diets without ethionine. Total non-essential acids and total amino acids were also lower at 2 and 4 weeks with methionine supplementation of ethionine-containing diets at both levels of SBTI. At 2 weeks, total amino acids levels were also lower in rats fed methionine supplemented diets with ethionine than in rats fed the two different levels of SBTI without ethionine. At 2 weeks, dietary methionine addition increased ratios of essential to total amino acids over those of rats fed the same amount of SBTI with ethionine but not to the higher level seen in rats fed SBTI without ethionine. At 4 weeks, the increase in essential to total amino acids occurring with methionine supplementation was significant only at the higher level of SBTI intake and, as for two weeks, did not achieve ratios seen in rats fed the two SBTI diets without ethionine.

Rats fed SBTI + ethionine-containing diets with supplementary cysteine had total essential amino acid levels similar to those of rats fed diets containing the two levels of SBTI with added ethionine and lower than those of rats fed diets containing SBTI alone. Total non-essential amino acids and total amino acids also remained at the high levels measured in rats fed SBTI with ethionine. Ratios of essential to total amino acids consequently remained at the low levels obtained in the SBTI + ethionine fed rats at both 2 and 4 weeks.

DISCUSSION

The equivalent weight gains and food intakes of rats fed the casein-based control diet and the two SBTI-containing diets without added ethionine, likely resulted from the presence of sufficient choline and methionine in all three diets to meet rat growth requirements. The use of highly unsaturated soy oil as the lipid source further served to prevent potential growth

problems resulting from any SBTI-induced lipotrope deficit. Rogers (1983) reported that the presence of dietary saturated fats greatly increases the severity of lipotrope deficits in rats.

The reduced weight gain in rats fed SBTI and ethionine, as also seen in the previous experiment, was the first indication noted of a potential lipotrope deficit. Growth deficits were not simply because of reduced food intake since they continued to be demonstrated in SBTI + ethionine-fed rats when weight gains were corrected for differences in feed intake by determination of feed efficiencies. Greatest deficits in weight gain and feed efficiency, relative to control rats, occurred in the first week of the feeding trial, although deficits remained significant after 4 weeks at both levels of SBTI. An adaptation to the negative growth effects of SBTI and ethionine ingestion may have occurred, perhaps reflecting decreased sulfur amino acid requirements of the older animals or enhanced metabolic detoxification of ethionine.

Supplementary methionine appeared to protect against the negative growth effects of dietary SBTI and ethionine, although methionine-supplemented rats at the higher level of dietary SBTI, never achieved weight gains equivalent to those of the control fed rats. This finding suggests that despite theoretical lipotrope adequacy, increasing dietary SBTI increased the negative growth effects of DL-ethionine. The general ineffectiveness of dietary cysteine in ameliorating the growth deficits suggests that the growth effects of SBTI and ethionine ingestion involve aspects of methionine metabolism distinct from those also involving cysteine. Brada et al. (1976) reported both 0.3% and 0.9% supplementary methionine to be only slightly effective at restoring growth of rats fed 0.3% ethionine. The considerably greater improvement obtained in the current study likely resulted from the lower (0.2%) level of ethionine used, and the avoidance of potential toxicity problems of higher doses of methionine (Harper et al. 1970).

The equivalent liver weights of casein-fed and SBTI-fed rats, as for weight gains and feed efficiencies, indicated the adequacy of the three diets for rat growth. The presence of ethionine resulted in a drop in absolute liver weight, reflecting the depressed growth of these animals. The higher relative liver weight measured at 2 weeks in SBTI + ethionine-fed rats compared to rats fed the high SBTI diet without ethionine may indicate the influence of ethionine on liver lipid deposition and protein synthesis. Ethionine causes accumulation of liver lipids (Farber 1967) likely resulting from effects on normal transport, deposition and metabolism of lipids. Dietary deficits of lipotropes, as for the induced deficit observed here in response to intakes of SBTI and ethionine, similarly cause excess liver lipid deposition (Ghoshal et al. 1983, Boyd et al. 1985).

The higher relative liver weights measured in rats fed supplementary methionine may result from the effects of dietary ethionine and its derivatives on liver methionine metabolism and intimately connected lipid metabolism. Finkelstein et al. (1982b) reported rapid clearance of injected methionine from normal rat liver tissues and maintenance of high levels of the transmethylation derivatives, S-adenosylmethionine and S-adenosylhomocysteine. The competition of ethionine and its activated derivative, S-adenosylethionine in the normal transmethylation reactions of methionine may have resulted in decreased methionine removal via this route, decreasing normal methylation reactions and resulting in excess methionine in liver tissues. The failure of cysteine supplementation to increase relative weight may reflect its inability to significantly affect methionine levels via influence on the transmethylation-transsulfuration pathway. Cysteine is an end product of transsulfuration and is rapidly converted to taurine and other derivatives such as glutathione (Tateishi et al. 1977).

The pancreatic trophic effects of SBTI were clearly shown at both 2 and 4 weeks by the higher absolute and relative pancreatic weights of rats fed increasing SBTI without ethionine.

The decrease in absolute and relative pancreatic weight with presence of dietary ethionine likely reflects the toxic influence of ethionine on the pancreas, since ethionine has been reported to cause necrosis of pancreatic tissues (Farber 1967). Competition of ethionine and derivatives in the transmethylation-transsulfuration pathway could also decrease conversion of methionine to cysteine, decreasing cysteine supply or availability and inhibiting pancreatic response to dietary SBTI. The pancreatic synthesis, storage and release of cysteine-rich proteases are major features of the pancreatic trophic response occurring in rats fed SBTI (Rackis and Gumbmann 1981).

The significantly higher relative pancreatic weights of rats fed methionine- compared to cysteine-supplemented diets (significant only at 4 weeks), suggests that decreased availability of cysteine is not a major factor in the lack of pancreatic trophic response in rats fed SBTI and ethionine.

Decreased absolute kidney and pancreatic weights in response to dietary SBTI combined with ethionine reflects the decreased growth of these animals relative to those fed diets in which ethionine was absent. As for absolute liver weights and rat growth in general, supplementary methionine overcame decreases whereas cysteine did not, likely reflecting the influence of dietary methionine on ethionine metabolism (Brada et al. 1976). The higher relative kidney weight of rats fed diets containing ethionine seen at 2 weeks may result from the kidney calcinosis observed in these animals. The failure to observe microscopic indications of pancreatic hypertrophy or hyperplasia probably results from the overriding necrotic influence of short-term intakes of dietary ethionine on pancreatic tissue (Fitzgerald and Alvizouri 1952, Farber 1967). Higher absolute and relative pancreatic weights of rats fed SBTI without ethionine suggest that pancreatic trophic response to SBTI did occur.

The elevation in serum triglycerides and total cholesterol measured in rats fed the SBTI

+ ethionine-containing diets further support conclusions of a lipotrope deficit whereas glutamate pyruvate transaminase and glutamate oxaloacetate transaminase are indicators of liver damage. Boyd et al. (1985) reported similar elevation in these and other serum markers of hepatic lipid accumulation and injury in rats fed a diet deficient in both choline and methionine. Changes in transmethylation reactions involved in lipid biosynthesis in liver cells isolated from lipotrope deficient rats have also been reported (Pascale et al. 1982).

Serum urea nitrogen, not measured by these authors was also higher in the current study in SBTI + ethionine-fed rats, although this may reflect lower protein quality of the ethionine-containing diets (Eggum 1970).

Supplementary dietary methionine effectively prevented the development of the clinical chemical symptoms of lipotrope deficit in the SBTI + ethionine-fed rats. The failure of cysteine to even partially moderate the high levels of serum lipids and enzymes would suggest its inadequacy as a supplement or protective agent under conditions of exposure to SBTI and ethionine.

Plasma free amino acid ratios have been useful for assessing the effects of processing on amino acid availability and protein digestibility in foods (Vaughan et al. 1977) and plasma or serum free amino acid levels have been used in assays of protein quality (McLaughlan 1963) and in predicting first limiting amino acids in protein mixtures (Sarwar et al. 1983). It has also been demonstrated that the achievement of dietary methionine adequacy for growth in rats is associated with changes in plasma levels of free essential amino acids and non-essential amino acids related to the transsulfuration pathway of methionine metabolism (Peace et al. 1986).

In the current study, an accumulation of essential amino acids because of unavailability of dietary methionine for protein synthesis in SBTI-containing diets was not supported by the

amino acid data. Serum threonine and arginine were higher at 2 weeks in SBTI-fed rats compared to casein controls but total essential amino acids showed no changes. The use of a combination of soy protein isolate and raw soy and the provision of supplementary methionine in these diets likely moderated potential decreases in availability of amino acids because of the lower digestibility of the raw soy present. Increased serum serine and glycine compared to casein-fed rats at both 2 and 4 weeks, however, suggest accumulation of these amino acid substrates or precursors of the transsulfuration pathway and decreased methionine utilization via this route.

The lower levels of several individual essential amino acids in rats fed SBTI + ethionine-containing diets may reflect a general inanition because of the metabolic effects of ethionine and depressed food. The higher serum free methionine compared to casein-control and SBTI-fed rats suggests a decreased utilization of methionine via the transsulfuration pathway that accounts for the lipotrope deficit indicated by growth deficits and changes in clinical chemistry parameters. The decreased utilization of the transsulfuration pathway is further supported by maintenance of the higher serum serine concentrations seen in SBTI-fed rats as well as accumulation of serine precursors, glycine and alanine (Yamaguchi 1990).

Elevated serum ornithine and taurine compared to casein-fed or SBTI-fed rats was also noted. High ornithine levels may explain the induction of ornithine decarboxylase activity, decreased S-adenosylmethionine and increased polyamine biosynthesis occurring in animals fed lipotrope-deficient diets and in various types of chemically-induced neoplasia (Feo et al. 1985). The elevation in serum taurine probably does not reflect increased biosynthesis via the transsulfuration pathway but rather its decreased utilization in lipid transport resulting from the ethionine-induced problems in liver lipid metabolism (Farber 1967).

The higher serum methionine in rats fed SBTI + ethionine diets with supplementary

methionine may reflect continued compromise of the transmethylation-transsulfuration pathway because of the combination of dietary SBTI and ethionine, and the failure of methionine clearance via this route. Levels of alanine and glycine were significantly lower compared to rats fed SBTI + ethionine diets, suggesting some amelioration of transsulfuration utilization. A decrease in SBTI + ethionine-induced lipotrope deficit is further suggested by the normalization of serum clinical chemistry parameters which have already been mentioned.

The continued high taurine levels suggested that taurine utilization in lipid metabolism and transport continued to be blocked in the methionine-supplemented rats.

The lower levels of most individual essential amino acids, total essential amino acids and decreased ratio of essential to total amino acids in rats fed cysteine-supplemented, SBTI + ethionine diets compared to rats fed the casein-control diet were similar to results obtained in rats fed SBTI + ethionine diets without any supplement. This finding would suggest that cysteine was not effective at normalizing serum free amino acid changes resulting from SBTI and ethionine ingestion. The continued elevation of glycine, alanine and serine in these animals, at concentrations greater than those seen in the methionine-supplemented rats, suggested that cysteine was not effective at enhancing deficits in the transsulfuration pathway induced by the lipotropic effects of SBTI and ethionine.

It has thus been demonstrated that combination of SBTI and DL-ethionine in diets adequate for rat growth, do induce a lipotrope deficit as manifested by decreased growth, and increases in serum clinical indicators of both hepatic lipid deposition and injury. Changes in concentrations of serum free essential amino acids and in serum free non-essential amino acids related to the transsulfuration pathway of methionine metabolism further suggest lipotrope deficit.

The increased responses in many of the symptoms by feeding higher levels of SBTI

with the same level of ethionine suggest that SBTI can exacerbate the effects of ethionine-induced lipotrope deficit. The transsulfuration-transmethylation pathway of methionine metabolism, known to be influenced by lipotrope deficit, is suggested as a prime candidate in the mechanisms of the antinutritional and carcinogenic effects reported for soybean trypsin inhibitors.

Supplementary methionine appears considerably more effective than cysteine in preventing or ameliorating many of the symptoms of SBTI + ethionine-induced lipotrope deficit. This has implications in terms of potential sulfur amino acid supplementation of SBTI-containing diets, although further studies should be undertaken to elucidate the complex interactions between ethionine and soy protein.

TABLE 4 1 Composition of diets

Ingredient	Percent of diet
Protein (Casein[1], Soyprotein isolate[2]/ground raw soybeans[3])	20.0
L-Met, L-Thr, L-Trp, L-Glu[4]	+
DL-Eth, L-Met, L-Cys(e)[5]	±
AIN-76 Mineral mix[6]	3.5
AIN 76A Vitamin mix[6]	1.0
Choline bitartrate	0.2
Fibre[7]	5.0
Soybean oil[8]	15.0
Corn starch[9]	-55.3

[1] Casein: ANRC Casein (Humko Sheffield Chemical, Division of Kraft Inc., Norwich, NY).
[2] Soyprotein isolate (Supro 710, Ralston Purina, St. Louis, MO).
[3] Maple Arrow Soybeans (Hardy Seeds Ltd., Inkerman, Ont.).
[4] Amino acids added to make diets meet NRC (1978) requirements for rat growth and to make them isonitrogenous, (Sigma Chemical Co., St. Louis, MO).
[5] DL-ethionine added at 0.2% of diet to Diets 3, 4, 5, 7, 8, 9.
L-methionine supplement (3.35 mmol/100 g) to Diets 4 and 8.
L-cysteine supplement (3.35 mmol/100 g) to Diets 5 and 9, (Sigma Chemical Co., St. Louis, MO).
[6] American Institute of Nutrition (1977), purchased from ICN Biomedicals Inc., Cleveland, OH.
[7] Fibre includes crude fibre of raw soybean meal and Alphacel.
[8] Soybean oil includes oil present in raw soybeans.
[9] Canada Starch Co., Toronto, Ont.

TABLE 4.2 Growth parameters[1] of male rats fed SBTI[2] with and without DL-ethionine and supplementary sulfur amino acids

		Diet								
Dietary		1	2	3	4	5	6	7	8	9
SBTI[2]		0	421	421	421	421	775	775	775	775
DL-ethionine (%)		0	0	0 2	0 2	0 2	0	0 2	0 2	0 2
SAA supplement[3]		0	0	0	M	C	0	0	M	C
	Week									
Weight gain (g)	1	42±1^{c}	40±1^{c}	12±2^{e}	23±2^{b}	9±1^{e}	41±1^{c}	14±2^{e}	26±2^{c}	12±2^{e}
	2	55±2^{c}	55±3^{c}	33±2^{e}	43±1^{b}	34±2^{e}	52±2^{c}	37±2^{e}	42±1^{b}	36±2^{e}
	3	50±2^{d}	49±3^{d}	41±2^{ex}	49±2^{d}	31±2^{e}	49±2^{d}	35±3de	42±3^{c}	36±3^{dex}
	4	53±4^{e}	48±4de	34±2de	44±2^{cd}	29±3^{e}	53±2^{e}	32±2de	39±2^{cx}	28±3^{e}
Feed intake (g)	1	74±1^{e}	68±2^{e}	45±2de	55±2^{c}	42±1^{e}	73±2^{e}	44±2de	61±3^{e}	49±2^{e}
	2	108±2^{c}	109±3^{c}	73±4^{e}	90±1^{b}	74±3^{e}	111±2^{c}	76±3^{e}	91±2^{b}	77±3^{e}
	3	117±5^{b}	126±5^{a}	100±5^{bx}	111±3^{cd}	85±3^{e}	128±4^{a}	86±6^{e}	96±5de	88±6de
	4	123±4^{ab}	122±5^{ab}	98±5de	115±3^{cd}	87±4^{e}	129±4^{a}	93±4^{e}	106±5^{bx}	90±5^{e}
Feed efficiency (g gain/100 g food intake)	1	56 2±1 4^{a}	58 2±1 1^{a}	25 4±3 7de	41 2±2 3^{c}	20 7±2 8^{e}	55 7±1 0^{a}	29 5±3 6^{e}	41 9±1 4^{c}	24 5±3 4de
	2	50 6±1 1^{c}	50 3±1 1^{bx}	44 9±1 9^{e}	47 8±0 8^{abx}	45 0±1 3^{e}	46 3±1 2^{e}	48 5±1 4^{abx}	46 3±1 3^{e}	46 6±1 8de
	3	42 3±1 0^{cx}	38 4±1 0de	41 0±1 2^{abcd}	44 4±1 0^{c}	36 6±2 5^{e}	38 0±1 0de	39 9±1 9^{abx}	43 4±1 3^{ab}	40 2±0 8^{abcx}
	4	42 5±2 0	39 4±1 9de	34 4±0 9^{ex}	38 5±1 1^{cde}	32 6±2 3de	41 1±1 0^{ef}	34 6±2 3^{abcd}	36 6±0 7^{abcde}	30 2±1 6^{e}

Means ± SEM of 8 animals per group means with different superscripts in the same row differ significantly ($P<0.05$)
SBTI Soybean trypsin inhibitor measured as mg trypsin inhibited per 100 g diet
[3]SAA supplement M L-methionine (3 35 mmol/100 g diet) C L cysteine (3 35 mmol 100 g diet)

TABLE 4.3 Organ weights[1] of male rats fed SBTI[2] with and without DL-ethionine and supplementary sulfur amino acids for 2 weeks.

	Diet								
Dietary	1	2	3	4	5	6	7	8	9
SBTI[2]	0	421	421	421	421	775	775	775	775
DL ethionine (%)	0	0	0 2	0 2	0 2	0	0 2	0 2	0 2
SAA supplement[3]	0	0	0	M	C	0	0	M	C
Liver (g)	6 70±0 31^{b}	6 77±0 19^{b}	4 78±0 37^{a}	6 24±0 24^{b}	4 86±0 36^{a}	6 35±0 20^{b}	5 25±0 28^{a}	6.38±0 28^{b}	5 21±0 35^{a}
(g/100 g body wt)	4 27±0 13^{a}	4 29±0 07^{a}	4 59±0 20^{ab}	4 97±0 15^{b}	4 75±0 21^{b}	4 16±0 07^{a}	4 75±0 14^{b}	5 00±0.11^{b}	4 84±0 17^{b}
Pancreas (g)	0 74±0 04^{bc}	0 88±0 03^{cd}	0 56±0 09^{ab}	0 71±0 04^{bc}	0 47±0 07^{a}	1 03±0.08^{d}	0 61±0 08^{ab}	0 85±0 05^{cd}	0 63±0 05^{ab}
(g/100 g body wt)	0 47±0 02^{a}	0 56±0 02^{ab}	0 53±0 07^{ab}	0 56±0 03^{ab}	0 45±0 06^{a}	0 67±0 04^{b}	0 55±0 07^{ab}	0 67±0 05^{b}	0 58±0 03^{ab}
Kidneys (g)	1 31±0 03^{bc}	1 41±0 05^{c}	1 02±0 03^{a}	1 26±0 02^{b}	1 02±0 05^{a}	1 37±0.03^{c}	1 02±0 04^{a}	1 26±0 03^{b}	1 07±0 04^{a}
(g/100 g body wt)	0 84±0 02^{a}	0 89±0 01^{a}	0 99±0 04^{b}	1 00±0 02^{b}	1 00±0 03^{b}	0 90±0 02^{a}	0 92±0 02^{ab}	0 99±0 04^{b}	1 01±0 05^{b}
Spleen (g)	0 70±0 05^{a}	0 64±0 03^{ab}	0 36±0 08^{a}	0 53±0 03^{bc}	0 36±0 03^{a}	0 60±0 03^{cd}	0 39±0 04^{a}	0 50±0 02^{b}	0 36±0 03^{a}
(g/100 g body wt)	0 44±0 02^{c}	0 40±0 01^{bc}	0 35±0 01^{ab}	0 42±0 02^{c}	0 34±0 02^{ab}	0 39±0 02^{abc}	0 34±0 03^{ab}	0 39±0 02^{abc}	0 33±0 02^{a}

Means ± SEM of 8 animals per group, means with different superscripts in the same row differ significantly ($P<0.05$)
[2]SBTI Soybean trypsin inhibitor measured as mg trypsin inhibited per 100 g diet
[3]SAA supplement M, L-methionine (3 35 mmol/100 g diet), C, L-cysteine (3 35 mmol/100 g diet)

TABLE 4.4 Organ weights[1] of male rats fed SBTI[2] with and without DL-ethionine and supplementary sulfur amino acids for 4 weeks.

	Diet								
Dietary	1	2	3	4	5	6	7	8	9
SBTI[2]	0	421	421	421	421	775	775	775	775
DL-ethionine (%)	0	0	0 2	0 2	0 2	0	0 2	0 2	0 2
SAA supplement[3]	0	0	0	M	C	0	0	M	C
Liver (g)	11 8±0 57^{c}	11 16±0 65^{bc}	8 02±0 38^{a}	11 90±0 52^{c}	7 06±0.25^{a}	11.59±0.43^{c}	7 31±0 36^{a}	10 01±0 55^{b}	7 20±0 40^{a}
(g/100 g body wt)	4 53±0 11^{a}	4 31±0 10^{a}	4 33±0 15^{a}	5 23±0 09^{b}	4 43±0 09^{a}	4 39±0.07^{a}	4 36±0.11^{a}	4 97±0 07^{b}	4.35±0 12^{a}
Pancreas (g)	1.38±0.08^{bc}	1 61±0 10^{cd}	0 90±0 07^{a}	1.32±0 10^{b}	0 71±0 10^{a}	1 74±0 07^{d}	0 90±0 10^{a}	1 20±0.12^{b}	0 75±0.12^{a}
(g/100 g body wt)	0 53±0 03^{abc}	0 62±0 03^{cd}	0 48±0 03^{ab}	0 57±0 03^{bcd}	0 43±0 05^{a}	0 66±0 03^{d}	0 53±0 05^{abc}	0 59±0 04^{bcd}	0.43±0 04^{a}
Kidneys (g)	2 17±0 12^{cd}	2 14±0 11^{cd}	1 61±0 05^{a}	1 97±0 06^{bc}	1 40±0 05^{a}	2 30±0 08^{d}	1 50±0 06^{a}	1 86±0 08^{b}	1 44±0 11^{a}
(g/100 g body wt)	0 83±0 04	0 83±0 02	0 87±0 03	0 86±0 03	0 87±0 02	0 87±0 02	0.90±0 06	0 93±0 04	0 86±0 03
Spleen (g)	0 87±0 04^{d}	0 81±0 06^{d}	0 57±0 04^{b}	0 78±0 04^{cd}	0 46±0 01^{a}	0 85±0.04^{d}	0 52±0 04^{ab}	0 71±0 01^{c}	0 49±0 03^{ab}
(g/100 g body wt)	0 33±0 01^{bcd}	0 31±0 01^{abc}	0 30±0 01^{ab}	0 34±0 01^{cd}	0 28±0 01^{a}	0 32±0 01^{abcd}	0 30±0 01^{ab}	0 35±0 01^{d}	0 29±0 02^{a}

Means ± SEM of 8 animals per group, means with different superscripts in the same row differ significantly ($P<0.05$)
[2]SBTI Soybean trypsin inhibitor measured as mg trypsin inhibited per 100 g diet
[3]SAA supplement M, L-methionine (3 35 mmol/100 g diet), C L-cysteine (3 35 mmol/100 g diet)

TABLE 4.5 Serum clinical chemistry parameters[1] of male rats fed SBTI[2] with and without DL-ethionine and supplementary sulfur amino acids for 2 weeks.

	Diet								
Dietary	1	2	3	4	5	6	7	8	9
SBTI[2]	0	421	421	421	421	775	775	775	775
DL-ethionine (%)	0	0	0.2	0.2	0.2	0	0.2	0.2	0.2
SAA supplement[3]	0	0	0	M	C	0	0	M	C
Cholesterol (mg/dL)	84±5[a]	84±7[a]	134±13[b]	82±5[a]	140±8[b]	78±5[a]	136±7[b]	83±3[a]	144±4[b]
Triglycerides (mg/dL)	320±23[a]	321±14[a]	915±86[b]	379±32[a]	980±92[b]	327±23[a]	956±51[b]	313±14[a]	1022±98[b]
Urea nitrogen (mg/dL)	16.6±1.3[a]	18.3±1.2[a]	22.6±1.1[b]	15.8±0.8[a]	22.2±2.1[b]	16.8±1.4[a]	23.7±2.4[bc]	17.6±0.9[a]	26.7±1.5[c]
Glutamate pyruvate transaminase (IU/mL)	33±1[ab]	31±1[ab]	51±5[d]	29±3[ab]	50±4[d]	37±3[bc]	48±5[cd]	26±1[a]	48±5[cd]
Glutamate oxaloacetate transaminase (IU/mL)	110±4[a]	117±5[ab]	146±14[c]	107±4[a]	135±7[bc]	109±3[a]	140±15[c]	100±8[a]	142±16[c]

[1]Means ± SEM of 8 animals per group. means with different superscripts in the same row differ significantly ($P<0.05$)

[2]SBTI Soybean trypsin inhibitor measured as mg trypsin inhibited per 100 g diet

[3]SAA supplement M, L-methionine (3.35 mmol/100 g diet), C, L-cysteine (3.35 mmol/100 g diet).

TABLE 4.6 Serum clinical chemistry parameters[1] of male rats fed SBTI[2] with and without DL-ethionine and supplementary sulfur amino acids for 4 weeks.

	Diet								
Dietary	1	2	3	4	5	6	7	8	9
SBTI[2]	0	421	421	421	421	775	775	775	775
DL-ethionine (%)	0	0	0 2	0 2	0 2	0	0 2	0 2	0 2
SAA supplement[3]	0	0	0	M	C	0	0	M	C
Cholesterol (mg/dL)	89±4^{cd}	81±4^{bcd}	94±8^{d}	63±6^{a}	97±5^{d}	77±4^{abc}	96±9^{d}	72±5^{ab}	83±6^{bcd}
Triglycerides (mg/dL)	243±16^{a}	254±23^{a}	753±31^{bc}	323±47^{a}	690±43^{b}	295±29^{a}	864±93^{c}	301±32^{a}	734±39^{bc}
Urea nitrogen (mg/dL)	20 1±1 1^{bc}	16 5±0 8^{ab}	20 2±1 3^{bc}	19 7±2 4^{bc}	17 5±0 7^{abc}	15 0±1 2^{a}	21.3±1 5^{c}	19 0±0.8^{abc}	17.0±1 2^{ab}
Glutamate pyruvate transaminase (IU/mL)	18±1^{a}	20±1^{a}	46±6^{b}	24±2^{a}	49±5^{bc}	23±1^{a}	58±6^{c}	24±1^{a}	46±3^{b}
Glutamate oxaloacetate transaminase (IU/mL)	78±2^{a}	79±3^{a}	113±11^{b}	75±4^{a}	114±4^{b}	84±5^{a}	109±6^{b}	72±3^{a}	101±6^{b}

Means ± SEM of 8 animals per group, means with different superscripts in the same row differ significantly ($P<0.05$)

[1]SBTI Soybean trypsin inhibitor measured as mg trypsin inhibited per 100 g diet

[3]SAA supplement M, L-methionine (3 35 mmol/100 g diet) C, L-cysteine (3 35 mmol/100 g diet)

TABLE 4.7 Serum free essential amino acids concentrations[1,2] in male rats fed SBTI[3] with and without DL-ethionine and supplementary sulfur amino acids for 2 weeks.

	Diet									
Dietary	1	2	3	4	5	6	7	8	9	SEM[4]
SBTI[3]	0	421	421	421	421	775	775	775	775	
DL-ethionine (%)	0	0	0.2	0.2	0.2	0	0.2	0.2	0.2	
SAA supplement[3]	0	0	0	M	C	0	0	M	C	
Arg	16.3^{bc}	27.3^{e}	7.0^{a}	17.7^{c}	13.9^{bc}	26.7^{e}	15.2^{bc}	22.1^{d}	12.9^{b}	1.3
His	13.5^{b}	11.3^{a}	13.8^{bc}	13.9^{bc}	15.1^{cde}	14.6^{bcd}	13.8^{bc}	15.5^{de}	16.4^{e}	0.5
Ile	11.9^{b}	13.1^{b}	12.7^{b}	9.5^{a}	13.6^{b}	13.5^{b}	11.9^{b}	9.5^{a}	13.1^{b}	0.6
Leu	17.1^{cd}	17.0^{cd}	16.2^{c}	11.6^{a}	16.7^{cd}	18.3^{d}	14.3^{b}	11.3^{a}	16.6^{cd}	0.6
Met	7.8^{ab}	7.5^{ab}	10.7^{bc}	13.2^{cde}	10.4^{bc}	5.8^{a}	12.3^{cd}	16.3^{e}	14.6^{de}	1.1
Phe	7 4^{a}	7.7^{a}	9.9^{c}	9.2^{bc}	10 0^{c}	8.0^{ab}	9.3^{c}	7.5^{a}	10.3^{c}	0.4
Thr	76.8^{c}	93.2^{d}	66.0^{bc}	40.1^{a}	67.2^{bc}	96.0^{d}	54.2^{ab}	39.7^{a}	60.4^{bc}	5.4
Trp	6 5^{ab}	6.7^{ab}	7.9^{c}	6.8^{b}	6.4^{ab}	5.8^{a}	8.2^{c}	6.6^{ab}	8.1^{c}	0.3
Val	24 6^{d}	21.5^{bc}	22.3^{cd}	15.8^{a}	23 3^{cd}	22.1^{cd}	19.2^{b}	14.5^{a}	21 7^{bcd}	0.9
Lys	65.8^{d}	52.4^{c}	29.8^{a}	53 7^{c}	32 4^{ab}	53.0^{c}	37.1^{b}	50.8^{c}	33 2^{ab}	2.1

[1] mol/100 mL serum.
[2] Means with different superscripts in the same row differ significantly ($P<0.05$)
[3] SBTI Soybean trypsin inhibitor measured as mg trypsin inhibited per 100 g diet
[4] SEM calculated from analysis of variance data
[5] SAA supplement: M, L-methionine (3 35 mmol/100 g diet); C, L-cysteine (3 35 mmol/100 g diet).

TABLE 4.8 Serum free essential amino acids concentrations[1,2] in male rats fed SBTI[3] with and without DL-ethionine and supplementary sulfur amino acids for 4 weeks

	Diet									
Dietary	1	2	3	4	5	6	7	8	9	SEM[4]
SBTI[3]	0	421	421	421	421	775	775	775	775	
DL-ethionine (%)	0	0	0.2	0 2	0 2	0	0.2	0 2	0 2	
SAA supplement[5]	0	0	0	M	C	0	0	M	C	
Arg	17 3^{b}	27 1^{d}	18.1^{b}	21.1^{c}	14 2^{a}	26 3^{d}	17 2^{b}	24 4^{d}	19.5^{bc}	0.9
His	10.9^{ab}	10.4^{a}	12.7^{abc}	16 4^{cd}	11 7^{ab}	11.1^{ab}	13.0^{abc}	18.4^{d}	14.6^{bc}	1.2
Ile	14 0^{e}	12.3^{d}	12.2^{cd}	9.8^{a}	12.7^{d}	12.1^{cd}	11.3^{bc}	10 4^{ab}	11 3^{bc}	0.3
Leu	21.3^{e}	19 0^{d}	16.4^{bc}	14.8^{ab}	17.9^{cd}	17.7^{cd}	14 2^{a}	15 6^{ab}	15 2^{ab}	0.6
Met	8.9^{ab}	8 2^{ab}	12.0^{c}	15.1^{d}	10.1^{bc}	6.7^{a}	7.6^{ab}	15.1^{d}	7.0^{a}	0.8
Phe	8 7^{ab}	9 0^{ab}	13 8^{d}	10 7^{c}	12 7^{d}	8.3^{a}	12.8^{d}	10.2^{bc}	10.8^{c}	0.5
Thr	95.0^{e}	92 9^{e}	54.1^{b}	54 7^{b}	59 2^{bc}	101.0^{e}	68.3^{cd}	42.9^{a}	77.2^{d}	3.2
Trp	10 8^{e}	10 1^{cde}	9.1^{bc}	10.3^{cde}	8 1^{ab}	9 2^{bc}	9 5^{cd}	10 5de	7.8^{a}	0 4
Val	30 9^{c}	22 9^{b}	22 1^{b}	19 1^{a}	23 5^{b}	22 2^{b}	19 0^{a}	18.8^{a}	19.5^{a}	0.7
Lys	53 3^{c}	42 5^{a}	46 8^{ab}	46 0^{ab}	41 9^{a}	44 2^{ab}	44 6^{ab}	49 5^{bc}	42 0^{a}	1.9

[1] μmol/100 mL serum
[2] Means with different superscripts in the same row differ significantly ($P<0.05$)
[3] SBTI Soybean trypsin inhibitor measured as mg trypsin inhibited per 100 g diet
[4] SEM calculated from analysis of variance data
[5] SAA supplement M, L-methionine (3 35 mmol/100 g diet), C, L-cysteine (3 35 mmol/100 g diet)

TABLE 4 9 Serum free non-essential amino acids[1] in male rats fed SBTI[2] with and without DL-ethionine and supplementary sulfur amino acids for 2 weeks.

	Diet									
Dietary	1	2	3	4	5	6	7	8	9	SEM[3]
SBTI[2]	0	421	421	421	421	775	775	775	775	
DL-ethionine (%)	0	0	0.2	0 2	0 2	0	0 2	0 2	0.2	
SAA supplement[4]	0	0	0	M	C	0	0	M	C	
Ala	83 1^{ab}	77.6^{a}	99.2^{c}	77.2^{a}	94.5^{bc}	87.5^{abc}	99.1^{c}	82.0^{ab}	97.2^{c}	4.1
Asp	3.4^{ab}	4 3^{bc}	4.6^{c}	4 3^{bc}	5.1^{c}	3.3^{a}	4.9^{c}	3.5^{ab}	4.6^{c}	0.3
Asn	7.9^{a}	9.0^{a}	12.6^{b}	8 8^{a}	13 4^{b}	9.2^{a}	13.2^{b}	9.3^{a}	15.7^{c}	0.7
Glu	15.6^{de}	16.7^{e}	13.6^{bc}	11.3^{a}	13.3^{bc}	14.5^{bcd}	14.8^{cd}	12.9^{ab}	12.9^{ab}	0.6
Gln	129.6^{b}	107.2^{a}	135.1^{b}	108.9^{a}	148.0^{c}	131.8^{b}	141.2^{bc}	108.5^{a}	133.0^{b}	4.1
Gly	36.8^{a}	63.6^{b}	114.3^{fg}	92.0^{cde}	101.9^{def}	73.5^{bc}	105.1^{ef}	84.0^{cd}	131.2^{g}	6.5
Pro	50.2^{c}	35.8^{a}	44.2^{bc}	29.7^{a}	42.2^{b}	34.1^{a}	43.7^{bc}	32.6^{a}	45.4^{bc}	2.1
Ser	45.8^{a}	62.5^{bc}	60.9^{bc}	41.7^{a}	63.8^{bc}	68.1^{c}	57.2^{b}	38.2^{a}	63.4^{bc}	2.5
Tau	10.1^{a}	14.7^{a}	31.5^{b}	41.4^{c}	41.5^{c}	13.6^{a}	30.9^{b}	40.4^{c}	39.9^{c}	1.7
Cit	13 3^{ab}	13 4^{ab}	21.6^{c}	11.4^{a}	20.0^{c}	15.9^{b}	19.9^{c}	13.0^{ab}	21.8^{c}	1.0
Orn	12.5^{a}	14 9^{a}	36.6^{c}	16.4^{a}	27.2^{b}	16.0^{a}	28.0^{b}	16.1^{a}	29.6^{b}	2.1
-Aba	1 8^{b}	1 5^{a}	2.0^{c}	1 6^{ab}	2 3^{d}	1 5^{a}	2.0^{c}	1.6^{ab}	1.7^{ab}	0 1

[1] mol/100 mL serum, means with different superscripts in the same row differ significantly ($P<0.05$).
[2] SBTI: Soybean trypsin inhibitor measured as mg trypsin inhibited per 100 g diet.
[3] SEM calculated from analysis of variance data.
[4] SAA supplement: M, L-methionine (3 35 mmol/100 g diet); C, L-cysteine (3 35 mmol/100 g diet)

TABLE 4.10 Serum free non-essential amino acids[1] in male rats fed SBTI[2] with and without DL-ethionine and supplementary sulfur amino acids for 4 weeks.

	Diet									
Dietary	1	2	3	4	5	6	7	8	9	SEM[3]
SBTI[2]	0	421	421	421	421	775	775	775	775	
DL-ethionine (%)	0	0	0.2	0 2	0.2	0	0.2	0.2	0.2	
SAA supplement[4]	0	0	0	M	C	0	0	M	C	
Ala	84.6^{c}	74.5^{ab}	94.5^{d}	73.3^{a}	92.3^{d}	82.3^{bc}	95.5^{d}	77.3^{abc}	95.1^{d}	2.6
Asp	3.5^{ab}	3.7^{bc}	4.8^{d}	3.0^{a}	4.2^{cd}	3.3^{ab}	4.6^{d}	3.0^{a}	4.2^{cd}	0.2
Asn	8.6^{a}	9.1^{abc}	11.4^{d}	8.6^{a}	11.4^{d}	9.6^{abc}	10.1^{bc}	8.9^{ab}	10.2^{c}	0.4
Glu	17.1^{d}	19.6^{e}	14.4^{bc}	12.4^{a}	15.3^{c}	21.3^{f}	13 4^{ab}	12.7^{a}	12.8^{a}	0.5
Gln	120.3^{ab}	106.2^{a}	133.4^{bc}	101.9^{a}	141.3^{c}	114.3^{a}	136.0^{bc}	104.0^{a}	143.9^{c}	5.8
Gly	40.1^{a}	58.7^{b}	103.0^{cd}	109.8^{d}	95.1^{c}	63.7^{b}	100.8^{cd}	102.6^{cd}	96.9^{cd}	4.4
Pro	61.3^{d}	34.9^{a}	46.7^{c}	33.6^{a}	41.1^{b}	35.2^{a}	43.2^{b}	36.0^{a}	41.3^{b}	0.9
Ser	49.3^{abc}	61.4^{d}	56.8^{d}	47.6^{ab}	55.8^{cd}	69.6^{e}	54.7^{bcd}	47.0^{a}	49.5^{abc}	2.3
Tau	17.2^{a}	18.8^{a}	24.7^{b}	41.1^{d}	37.0^{c}	16.8^{a}	25.1^{b}	37.6^{c}	41.0^{d}	0.8
Cit	12 7^{ab}	12.0^{ab}	15.5^{c}	10 9^{a}	20.3^{d}	12.6^{ab}	20.2^{d}	14.2^{bc}	21.6^{d}	0.7
Orn	11.7^{a}	13 4^{ab}	29.2^{d}	14.5^{b}	31.3^{d}	13.8^{ab}	30.8^{d}	14.6^{b}	26.1^{c}	0.7
-Aba	1 2^{b}	0 8^{a}	1.9^{c}	1.3^{b}	2 2^{d}	1.2^{b}	1.5^{b}	1.3^{b}	1.9^{c}	0 1

[1] mol/100 mL serum, means with different superscripts in the same row differ significantly ($P<0.05$).
[2] SBTI: Soybean trypsin inhibitor measured as mg trypsin inhibited per 100 g diet
[3] SEM calculated from analysis of variance data.
[4] SAA supplement M, L-methionine (3 35 mmol/100 g diet), C, L-cysteine (3 35 mmol/100 g diet).

TABLE 4.11 Serum free amino acid parameters of male rats fed SBTI[1] with and without DL-ethionine and supplementary sulfur amino acids for 2 weeks.

	Diet									
Dietary	1	2	3	4	5	6	7	8	9	SEM[2]
SBTI[1]	0	421	421	421	421	775	775	775	775	
DL-ethionine (%)	0	0	0.2	0.2	0.2	0	0.2	0.2	0.2	
SAA supplement[3]	0	0	0	M	C	0	0	M	C	
Total essential[4] amino acids	262^{d}	270^{d}	219^{bc}	198^{a}	230^{c}	274^{d}	219^{bc}	201^{ab}	229^{c}	6.4
Total non-essential[4] amino acids	417^{a}	427^{a}	583^{cd}	452^{ab}	581^{cd}	475^{b}	567^{c}	448^{ab}	603^{d}	11.2
Total[4] amino acids	679^{ab}	698^{b}	802^{d}	650^{a}	811^{d}	749^{c}	786^{cd}	650^{a}	832^{d}	15.1
Essential amino acids / Total amino acids	0.385^{d}	0.388^{d}	0.273^{a}	0.305^{b}	0 283^{a}	0.366^{c}	0.278^{a}	0.310^{b}	0.276^{a}	0.005

[1]SBTI: Soybean trypsin inhibitor measured as mg trypsin inhibited per 100 g diet.
[2]SEM calculated from analysis of variance data
[3]SAA supplement: M, L-methionine (3.35 mmol/100 g diet); C, L-cysteine (3.35 mmol/100 g diet).
[4]μ mol/100 mL serum, means with different superscripts in the same row differ significantly ($P<0.05$).

TABLE 4.12 Serum free amino acid parameters of male rats fed SBTI[1] with and without DL-ethionine and supplementary sulfur amino acids for 4 weeks.

	Diet									
Dietary	1	2	3	4	5	6	7	8	9	SEM[2]
SBTI[1]	0	421	421	421	421	775	775	775	775	
DL-ethionine (%)	0	0	0.2	0.2	0.2	0	0.2	0.2	0.2	
SAA supplement[3]	0	0	0	M	C	0	0	M	C	
Total essential[4] amino acids	286^{c}	268^{c}	250^{b}	226^{a}	235^{ab}	274^{cd}	240^{ab}	225^{a}	244^{ab}	6.0
Total non-essential[4] amino acids	434^{ab}	420^{a}	543^{c}	465^{b}	554^{c}	450^{ab}	543^{c}	466^{b}	552^{c}	12.7
Total[4] amino acids	720^{a}	688^{a}	793^{b}	691^{a}	790^{b}	724^{a}	783^{b}	691^{a}	796^{b}	17.5
Essential amino acids / Total amino acids	0.397^{e}	0.389^{de}	0.315^{bc}	0.326^{c}	0.298^{a}	0.379^{d}	0.307^{ab}	0.326^{c}	0.306^{ab}	0.005

[1]SBTI Soybean trypsin inhibitor measured as mg trypsin inhibited per 100 g diet.
[2]SEM calculated from analysis of variance data
[3]SAA supplement: M, L-methionine (3.35 mmol/100 g diet), C, L-cysteine (3.35 mmol/100 g diet).
[4]. mol/100 mL serum, means with different superscripts in the same row differ significantly ($P<0.05$).

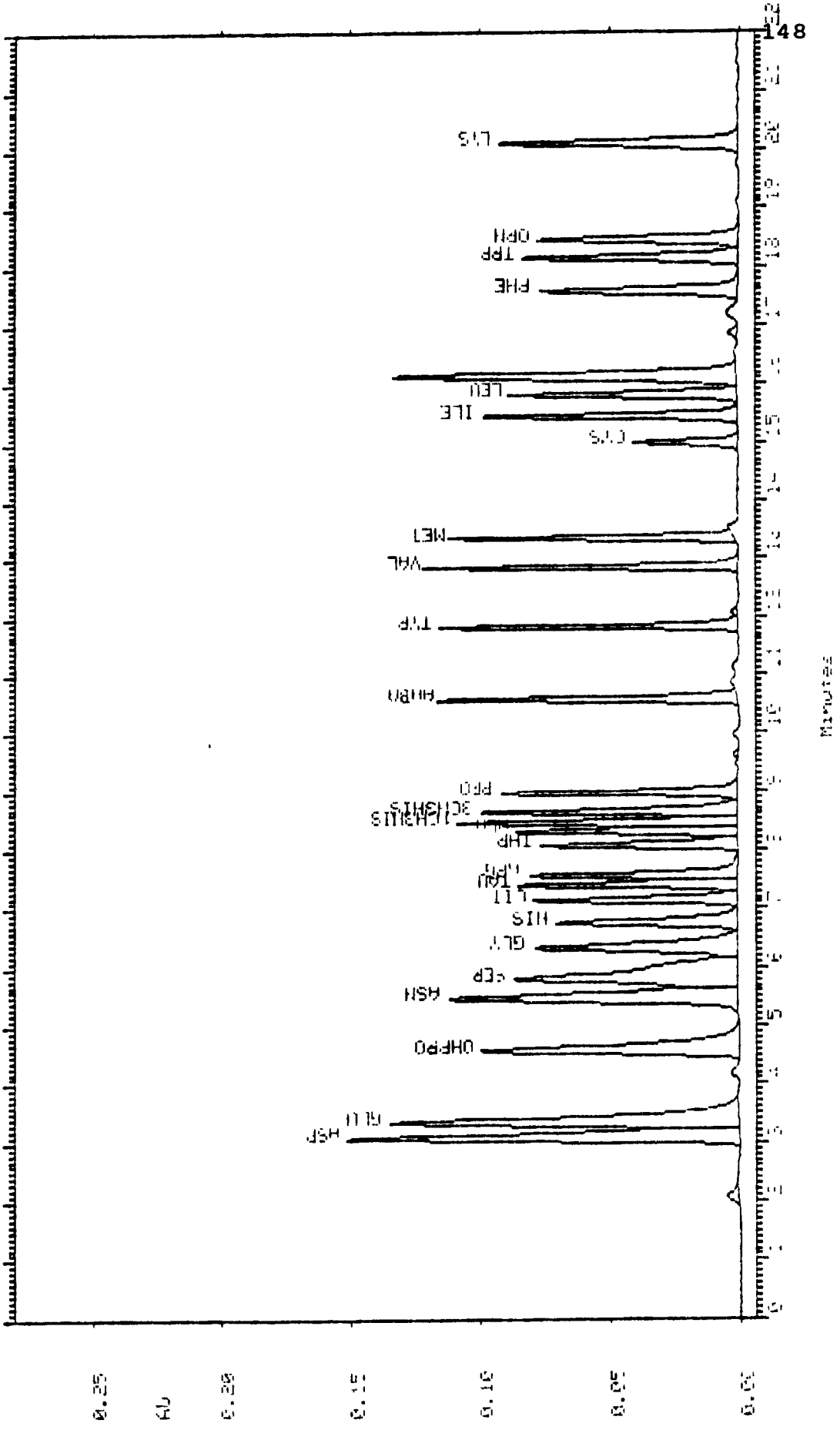
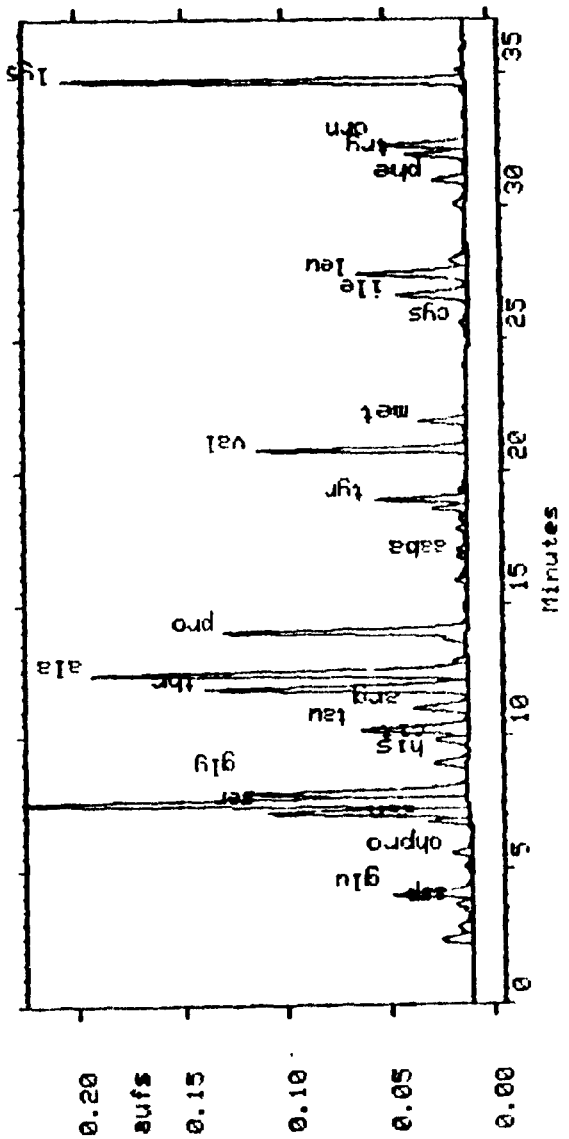


Figure 4.1 Elution profile of PTC-amino acid standard

Figure 4.2 Elution profile of rat serum PTC-amino acids



5. EFFECTS OF DIETARY SOYBEAN TRYPSIN INHIBITORS AND SUPPLEMENTARY SULFUR AMINO ACIDS ON HEPATIC LEVELS OF S-ADENOSYLMETHIONINE AND FREE AMINO ACIDS IN THE GROWING RAT

INTRODUCTION

Soybean trypsin inhibitors (SBTI) decrease protein digestibility and amino acid availability as well as reduce growth and cause pancreatic hypertrophy and hyperplasia when fed to susceptible animals. The pancreatic effects stem from loss of normal feedback inhibition on pancreatic proteinase synthesis and output resulting from proteinase inactivation by trypsin inhibitors in the gut. The increased synthesis and loss of endogenous protein exacerbates the problems of low digestibility of dietary proteins and decreased amino acid availability occurring with ingestion of the legume (Green and Lyman 1972, Schneeman and Lyman 1975, Rackis and Gumbmann 1981). The ingestion of soy products containing active trypsin inhibitors by rats has also been linked to the development of pancreatic cancer both in the presence and absence of chemical carcinogens (Levison et al. 1979, McGuinness et al. 1984, Gumbmann et al. 1986). The mechanism(s) of such effects remain speculative.

S-adenosylmethionine (SAM) is produced in the activation step of the transmethylation pathway of methionine metabolism. SAM serves as the major source of methyl groups for most biological methylation reactions and the metabolism of its homocysteine derivative via the subsequent transsulfuration pathway can give rise to other important amino acids such as taurine and cysteine. The transmethylation-transsulfuration sequence is the major pathway for methionine metabolism in mammalian liver (Finkelstein 1990). S-adenosylhomocysteine (SAH), an intermediate in the sequence, acts as an inhibitor of most methylation reactions.

SAM by itself can also act as an inhibitor of its own synthesis (Oden and Clarke 1983, Hoffman 1984).

The essential amino acid methionine, is one of a class of compounds termed lipotropes that are essential for normal lipid metabolism. A deficiency of dietary lipotropes (which also includes choline, vitamin B_{12} and folate), can decrease normal methylation reactions leading to development of fatty liver and kidney hypertrophy (Lombardi 1971, Newberne et al. 1968). Lipotrope deficits have also been implicated in the development of several cancers (Yokoyama et al. 1985, Mikol et al. 1983, Rogers 1983). Inadequate intakes of dietary lipotropes are reported to cause lower levels of hepatic SAM and increase in SAH (Shivapurkar and Poirier 1983) and the concentration of SAM and the ratio of SAM/SAH have been used as indices of methyl group availability (Hoffman et al. 1978, Henning et al. 1989).

Ethionine, the hepatocarcinogenic analogue of methionine, competes with methionine in the transmethylation-transsulfuration pathway, using up ATP and lowering the effective level of SAM. It has been suggested that the resultant decrease in available methyl groups induces hypomethylated DNA thereby enhancing abnormal DNA expression and resulting in carcinogenesis (Hyde and Poirier 1982, Shivapurkar et al. 1984).

The effects of various dietary and chemical treatments on tissue levels of SAM and SAH have been reported by several authors (Eloranta et al. 1976, Finkelstein et al. 1982a, 1982b, 1988, Loo and Smith 1986, Henning et al. 1989). Because of the known effects of SBTI on amino acid availability and the capacity of supplementary sulfur amino acids to moderate some of the antinutritional effects of SBTI (Gumbmann and Friedman 1987), it was of interest to see if the transmethylation-transsulfuration pathway was involved in the mechanism of trypsin inhibitor action. Combination of SBTI with DL-ethionine might be expected to enhance any changes that occurred as a result of SBTI alone. This finding could

point to similarities in the action of the two dietary factors and to another aspect of the mechanism whereby SBTI induce antinutritional effects and over the longer term initiate or potentiate carcinogenesis.

The sulfur amino acid cysteine is known to be sparing of dietary methionine requirements (Shannon et al. 1972, Stipanuk and Benevenga 1977). Because of the interest in methionine/cysteine ratios in terms of dietary sulfur amino acid requirements of infants and in supplementation of infant formulas (Zlotkin and Anderson 1982, Fomon et al. 1986) it was also of interest to investigate the potential influence of these two amino acids on transmethylation-transsulfuration metabolites when used as supplements in rat diets containing trypsin inhibitors and ethionine.

MATERIALS AND METHODS

Weanling male Wistar rats were used in two separate experiments as previously described in Sections III. 3 and III. 4.

In the first experiment, a total of 216 rats were placed in individual stainless steel, mesh-bottomed cages in a randomized complete block design involving 24 replicates in which each of 9 test diets was represented once per block. Blocking was on the basis of matched initial body weights. Diet composition and details of animal treatment have been described in Section III. 3. The diets contained 20% dietary protein and provided one of three levels of SBTI (0, 447, 808 mg trypsin inhibited per 100 g diet) and one of three levels of DL-ethionine (0, 0.05% and 0.10%).

In the second experiment, a total of 144 rats were placed in individual stainless steel, mesh-bottomed cages in a randomized complete block design involving 16 replicates in which

each of 9 test diets was represented once per block. Blocking was on the basis of matched initial body weights. Diets and animal treatment have been described in Section III. 4. The diets contained 20% dietary protein and provided two different levels of soybean trypsin inhibitor (405 and 775 trypsin inhibited per 100 g diet) with and without 0.2% DL-ethionine. Additional amino acids were added to make diets matching in essential amino acids and to ensure they met rat growth requirements (National Research Council 1978). Similar trypsin inhibitor + ethionine diets were formulated with and without equimolar (3.35 mmol/100g) diet supplements of L-methionine or L-cysteine. An additional casein-based diet, meeting rat growth requirements, without trypsin inhibitors or DL-ethionine present, was also formulated. All diets were made isonitrogenous by suitable addition of L-glutamic acid.

In the first experiment, diets and tap water were provided ad libitum for up to 12 weeks and in the second experiment for up to 4 weeks. After 4, 8 and 12 weeks in the first experiment and after 2 and 4 weeks in the second experiment, food was removed early in the morning from rats in the same 8 replicates. After 1 hour, rats were placed under halothane anaesthesia, the livers exposed by a mid-ventral incision and a small portion of liver tissue obtained by a freeze-clamp technique using liquid nitrogen-chilled tongs. The weight of the frozen tissue was determined after transfer to a tared test tube containing 2.0 ml of 0.4 N perchloric acid (PCA) (0°C) and the tissue was homogenized using a Brinkman polytron (Brinkman Instruments, Rexdale, Ont.). The chilled perchloric acid homogenate was spun down at 1600 x g in an IEC CENTRA-7R refrigerated centrifuge (International Equipment Co., Damon Corp., Needham Heights, MA) at 5°C and the supernatant immediately frozen and stored at -70° C in small screw-topped sample vials. Immediately after removal of the freeze-clamped liver tissue, the anaesthetized animals were exsanguinated via the dorsal aorta and

blood and other tissues including the balance of the liver were collected and stored as previously described in Sections III.3 and III.4.

Adenosine, S-adenosyl-L-methionine (SAM), S-adenosyl-L-homocysteine (SAH), S-adenosyl-L-ethionine (SAE) were purchased from Sigma Chemical Co., St. Louis, MO). Ammonium formate and ammonium sulfate were from Fisher Scientific (Fair Lawn, NJ). Milli-Q purified water (Milli-Q-Water System, Millipore Corp., Bedford, MA) was used in preparation of all solutions and buffers.

Analysis of S-adenosyl-derivatives was by HPLC using a modification of a method described by Gordon et al. (1987). A Waters HPLC system consisting of 2 Model 510 pumps, a 710B Wisp autoinjector, a Model 481 UV Spectrophotometer, and an 840 Data System was used along with a Radial Compression Module (RCM 8 x 10) and an SCX Cation exchange Radial-Pak cartridge (8mm x 10 cm) (Waters Chromatography Div., Millipore Corp., Milford, MA).

A gradient elution method was employed using prefiltered (0.45μ HA filters, Millipore) and degassed ammonium formate buffers (Buffer A: 1.0mM NH_4COOH, pH 4.0 adjusted with 5% formic acid; Buffer B: 0.2M NH_4COOH, 0.8M $(NH_4)_2SO_4$, pH adjusted to 4.0 with concentrated and 5% formic acid). Flow rates were 2.5 mL per minute during gradient elution with increasing amounts of Buffer B using Water's Curve 6 throughout. Gradient conditions were: Initial 100% A: 0% B; 8 mins. 100% A: 0% B; 20 mins. 95% A: 5% B; 28 mins. 70% A: 30% B; 33 mins 0% A: 100% B; 37 mins. 0% A: 100% B; 39 mins. 100% A: 0% B; 52 mins 100% A: 0% B. This procedure permitted optimal separation of compounds of interest and sufficient time for column cleaning and regeneration. Compounds of interest eluted within 30 mins.

PCA extracts were thawed, filtered through 0.22 μ Millex-GS filters and 200 μL aliquots were applied to the column. The S-adenosyl compounds were identified and quantitated by comparison of retention times and peak area absorbance with known molar concentrations of standard compounds. Suitable dilutions of standard compounds were made in fresh-filtered 0.4 N perchloric acid and aliquots of a working standard were prepared, frozen at -70° C and thawed for daily use. Molar concentrations of commercial standards were corrected for stated purity provided by the supplier. No internal standard was included because the HPLC was calibrated daily with fresh thawed standard solution. Recovery of SAM and SAE were determined by comparison of concentrations of the compounds in spiked and unspiked samples of fresh, freeze-clamped liver tissue subjected to the same homogenization, centrifugation, freezing and filtering procedure prior to analysis Chromatographic profiles of standards and a representative liver extract are shown in Figure 5.1.

In the first experiment duplicate samples of frozen liver tissue were subjected to analysis for total lipids. In the second experiment equal weights of liver tissue from pairs of rats fed the same diet for the same time period were pooled and used for analysis of total lipids. Total lipid analysis was by the method of Bligh and Dyer (1959).

Liver tissues from the second experiment were also analyzed for concentrations of free amino acids. This involved precolumn phenylisothiocyanate (PITC) derivatization of freeze-dried, powdered liver samples that had first been deproteinized by vortexing in the presence of 0.1N HCl. Derivatives were separated using the same Waters HPLC system described above with an added Waters temperature control module. A Waters Pico-tag amino acid analysis column was used and derivatization, standards and separation conditions were exactly as described by Sarwar and Botting (1990). Because of analytical constraints, livers

of animals from only 4 treatment replicates at each time period were analyzed for free amino acids. This involved deproteinization, derivatization and analysis of duplicate liver samples from 4 animals on each diet treatment at both 2 and 4 weeks.

Statistical results are expressed as means ± SEM. For each experiment and time period, differences in concentrations of hepatic S-adenosyl derivatives, total hepatic lipid and selected hepatic free amino acids in rats fed the different test diets were compared by analysis of variance using the PCANOVA program (Human System Dynamics, Northridge, CA). Significant differences between means were determined by Duncan's Multiple Range Test (Duncan 1955).

RESULTS

Tables 5.1, 5.2 and 5.3 reveal the effects of increased levels of SBTI and DL-ethionine on liver concentrations of S-adenosyl-derivatives at 4, 8 and 12 weeks respectively. Figures 5.2, 5.3 and 5.4 graphically demonstrate the changes in SAM concentrations and in the ratios of the concentrations of SAM/SAH and SAE/SAM.

Hepatic concentrations of SAH appeared to be relatively stable. Presence of dietary SBTI without ethionine (Diets 2 and 3) resulted in some decreases in hepatic SAH relative to concentrations in rats fed the control diet without SBTI or ethionine (Diet 1). The lower levels however, were significantly different from those of control-fed rats only at the intermediate levels of SBTI intake at 4 and 12 weeks and at the highest level of dietary SBTI at 8 weeks. SAH concentrations in rats fed the intermediate (Diet 2) and high (Diet 3) levels of SBTI did not differ significantly from each other at any of the times measured. Significantly lower hepatic SAH concentration in rats fed ethionine without SBTI occurred only at 12 weeks at

the 0.05% level of ethionine (Diet 4). Rats fed SBTI and ethionine had lower concentrations of hepatic SAH relative to control-fed rats at 4 and 8 weeks at the highest level of SBTI with 0.05% ethionine and at the intermediate level of SBTI with 0.10% ethionine. At 12 weeks rats fed at the intermediate level of SBTI with 0.05% ethionine and at both intermediate and high levels of SBTI with 0.10% ethionine had lower levels of hepatic SAH compared to control fed rats.

Changes in hepatic SAM concentrations in response to the various dietary treatments were much more pronounced. After 4, 8 and 12 weeks, rats fed SBTI had lower levels of SAM relative to levels in control-fed rats. The lower levels of hepatic SAM consistently measured at each time period, in rats fed at the higher level of SBTI did not, however, differ significantly from those of rats fed the intermediate level of SBTI. Increasing dietary ethionine without SBTI resulted in even lower levels of hepatic SAM than measured in rats fed SBTI Rats fed combinations of SBTI and ethionine had the lowest levels of hepatic SAM and this was most evident at the 0.05% level of dietary ethionine.

Hepatic concentrations of SAE appeared to be dependent on level of dietary ethionine. No SAE was detected in liver extracts of rats fed diets without ethionine. At each time measured, SAE levels appeared not to be influenced by dietary SBTI. Only at 8 weeks at the highest level of ethionine fed, was hepatic SAE significantly higher when SBTI was combined with ethionine. Hepatic concentrations of SAE at the same level of dietary ethionine and SBTI appeared to decrease somewhat over the course of the experiment

The ratio of hepatic concentration of S-adenosylmethionine to S-adenosylhomocysteine (SAM/SAH) decreased with both increasing dietary SBTI without ethionine and with increasing ethionine without SBTI. Decreases were more pronounced with the ethionine-containing diets.

Combination of the two dietary factors at the intermediate level of ethionine tended to result in lower SAM/SAH ratios compared to ratios in rats fed either factor alone.

Hepatic SAE/SAM ratios in contrast, increased with increasing dietary SBTI especially at the 0.10% level of dietary ethionine. Increasing dietary ethionine also resulted in higher ratios of SAE/SAM and highest ratios were obtained at each time period with combinations of highest level of ethionine and SBTI. Ratios of SAE/SAM at the same combinations of SBTI and ethionine tended to moderate over the course of the experiment.

No consistent changes in liver adenosine levels (not shown) were detected with either increasing dietary SBTI or ethionine or combinations of the two factors at the levels employed at either 4, 8 or 12 weeks. Interference of an unknown compound during the HPLC separation of adenosine in liver extracts made accurate quantification difficult. There did appear to be a decrease in overall concentration in this compound over the three months of the study for each diet group.

Increasing dietary SBTI without ethionine resulted in lower hepatic lipid levels (Table 5.6) compared to controls, although differences at 8 weeks were not significant. Consistently lower lipid levels were measured in rats fed the higher compared to the lower level of SBTI although differences were not significant. Increasing dietary ethionine without SBTI resulted in increased liver lipid levels except at 4 weeks at the highest level of ethionine fed. Combination of SBTI and ethionine usually decreased liver lipid compared to levels in rats fed the same level of ethionine without SBTI. Liver lipid levels of rats fed each diet generally appeared to increase as the experiment continued.

In the second experiment, as shown in Tables 5.4 and 5.5, concentrations of hepatic SAH were not significantly different among the various diet groups. Concentrations of SAM

and ratios of SAM/SAH and SAE/SAM, as also shown in Tables 5.4 and 5.5 and in Figures 5.5 to 5.7, did vary.

Liver SAM concentrations were equivalent in rats fed the casein-based diet without SBTI or ethionine (Diet 1) and the two soy-based diets with increasing amounts of SBTI without ethionine (Diets 2 and 6) after 2 (Table 5.4) and 4 (Table 5.5) weeks. Rats fed ethionine at both levels of SBTI (Diets 3 and 7) had lower liver SAM concentrations than those fed SBTI alone, although this was not significant at 2 weeks. Addition of supplementary methionine prevented these lower levels while the equimolar cysteine supplement was not effective.

Rats fed the SBTI diets with added ethionine had levels of hepatic SAE higher than those of the first experiment probably because of the higher dose level used in the second experiment. Addition of supplementary methionine appeared to moderate SAE levels although this was significant only at the higher level of dietary SBTI at 4 weeks. Supplementary cysteine was not effective in moderating the high SAE levels occurring with combination of dietary SBTI and ethionine.

Hepatic ratios of SAM/SAH were equivalent in animals fed the casein-based diet without ethionine or the two soy-based diets without ethionine at both 2 and 4 weeks. Presence of dietary ethionine resulted in a drop in SAM/SAH relative to rats fed SBTI alone, although this was significant only at the higher level of SBTI at week 4. Rats fed the SBTI + ethionine diets with added methionine had higher ratios of liver SAM/SAH compared to rats fed SBTI and ethionine. Rats fed cysteine-supplemented SBTI + ethionine diets, however, retained lower ratios of SAM/SAH.

The high hepatic SAE/SAM ratios of rats fed both levels of SBTI with added ethionine at both 2 and 4 weeks were moderated with supplementary methionine. Supplementary cysteine had no such moderating effect.

Total hepatic lipid levels of rats fed SBTI and DL-ethionine with and without supplementary sulfur amino acids are shown in Table 5.7. At 2 weeks, increasing SBTI without ethionine (Diets 2 and 6) did not affect hepatic total lipid levels. At 4 weeks, as seen in the previous experiment, increased dietary SBTI caused a drop in total hepatic lipids compared to rats fed the casein-based diet without SBTI. At 2 weeks, at both levels of SBTI tested, the presence of 0.2% ethionine had no significant effect on total lipid levels. At 4 weeks, rats fed the combination of ethionine and the lower level of dietary SBTI had lower hepatic lipid levels compared to control-fed rats or rats fed the same level of SBTI without ethionine. Methionine supplementation resulted in higher hepatic lipid levels compared to rats fed diets containing SBTI and ethionine, at 2 weeks in rats fed the higher level of SBTI and at 4 weeks in rats fed the lower level of SBTI. Cysteine supplementation of SBTI + ethionine-containing diets resulted in higher total liver lipid levels compared to rats fed SBTI + ethionine-containing diets only at 2 weeks at the lower level of SBTI.

Tables 5.8 and 5.9 reveal the concentrations of selected free amino acids present in the liver tissues of rats fed SBTI and ethionine with and without supplementary L-methionine and L-cysteine. At 2 weeks, concentrations of free L-methionine did not differ in rats fed any of the diets. At 4 weeks, methionine levels were equivalent in rats fed the casein-based diet or the diets containing SBTI without ethionine. Presence of dietary ethionine resulted in higher free methionine levels at 4 weeks, which appeared to be moderated at the higher level of SBTI by both methionine and cysteine supplementation.

Liver free L-threonine concentrations were also equivalent at both 2 and 4 weeks in rats fed the casein-based diet and the two diets with increasing SBTI. Presence of dietary ethionine resulted in lower threonine compared to levels in rats fed SBTI diets only at 2 weeks at the higher level of SBTI. Methionine and cysteine supplementation at 2 weeks had no effect on threonine levels compared to rats fed diets containing SBTI and ethionine. At 4 weeks, methionine and cysteine supplementation resulted in significantly lower and higher levels respectively, of liver free threonine compared to rats fed ethionine combined with the highest level of SBTI.

Liver free L-lysine was also equivalent in rats fed the casein- and soy-based diets without ethionine but increased in response to dietary ethionine. Methionine supplementation partially prevented this increase at 4 weeks at the lower level of dietary SBTI whereas cysteine supplementation did not.

Changes in hepatic free non-essential amino acids related to the transsulfuration pathway are also shown in Tables 5.8 and 5.9. L-serine increased in response to increasing dietary SBTI at both 2 and 4 weeks, whereas levels of L-glycine, L-alanine and L-taurine did not.

The presence of dietary ethionine appeared to moderate the increase in serine at the higher level of dietary SBTI at 2 and 4 weeks. Glycine increased in response to combination of dietary SBTI and ethionine at both 2 and 4 weeks. Alanine also appeared to increase although this was significant only at 2 weeks at the highest level of SBTI fed. Taurine was also higher in rats fed SBTI + ethionine compared to rats fed SBTI without ethionine

Methionine supplementation of SBTI + ethionine diets, resulted in lower hepatic serine, glycine and alanine, with levels equivalent to those of rats fed the casein-based diet without

SBTI or ethionine. Taurine levels were also lower compared to rats fed SBTI + ethionine diets although this was significant only at the highest level of SBTI + ethionine at 4 weeks.

L-cysteine did not have the lowering effect on liver serine and glycine that was seen with supplemental methionine. Serine and glycine levels remained equivalent to those seen in rats fed SBTI + ethionine-containing diets. At 4 weeks, cysteine supplementation was also not effective at lowering L-alanine levels. Hepatic taurine levels were also higher in rats receiving SBTI + ethionine with supplemental cysteine.

DISCUSSION

The first experiment demonstrates that the presence of dietary SBTI can affect the transmethylation-transsulfuration pathway of methionine metabolism. The maximal decreases in hepatic SAM (50, 37, 34%) and in the SAM/SAH ratio (39, 24, 23%) at 4, 8 and 12 weeks respectively, in rats fed SBTI-containing diets, compared to rats fed the casein-based diet show that SBTI compromised the availability of methyl groups. The lower levels of hepatic SAH measured over the 12 weeks of the experiment may indicate decreased SAH synthesis with decreased SAM synthesis. The decreased availability of SAM appeared to be maintained with continued exposure to dietary SBTI, suggesting that SBTI can have an ongoing influence on the pathway .

A similar compromise of methyl group availability has been shown in studies of young or full grown rats fed diets deficient in methionine or choline (Finkelstein et al. 1982c, Zeisel et al. 1989). Henning et al. (1989) reported that induced, increased requirement for SAM-mediated methylation of nicotinamide or removal of folate from methionine limiting, choline-deficient diets also increased methyl donor deficits in rats. The current study would seem to

be the first report in which SBTI, a naturally occurring dietary antinutrient, has contributed to a similar and prolonged change in transmethylation-transsulfuration metabolites.

Finkelstein et al. (1982c) suggested that decreased methionine availability was the basis for lowered hepatic levels of methionine, SAM and betaine occurring in rats after 4 days consumption of a choline deficient diet that was marginally adequate in methionine. Decreased availability of choline-derived betaine was proposed to have limited methionine regeneration from homocysteine, leading to a decrease in available methionine, decreased SAM synthesis and accumulation of SAH.

Zeisel et al. (1989) found a limited decrease in liver methionine with prolonged (42 day) dietary choline deficit in rats. They suggested that hepatic methionine adenosyltransferase isoenzymes were sensitive to small changes in methionine concentration and that decreased hepatic SAM stemmed from its increased utilization as a substrate for phosphatidylcholine synthesis via sequential SAM-mediated methylation of phosphatidylethanolamine. Decreased availability of methionine resulting from the effects of increasing dietary SBTI was likely the reason for the decreased SAM and lower SAM/SAH measured in the current experiment

The detection of SAE, its dose-dependent increase, and the high ratio of SAE/SAM in response to dietary DL-ethionine without SBTI were expected Hyde and Poirier (1982) reported that 0.1 to 0.3% dietary ethionine fed to rats for up to 6 weeks resulted in a great decrease in hepatic SAM and a dose-dependent increase in the ratio of SAE/SAM At the levels used in the current experiment, dietary DL-ethionine appeared a much more potent influence on intermediates of the transsulfuration pathway than did SBTI Inhibition of SAM biosynthesis both by ethionine and by its S-adenosyl-derivative, SAE, was likely the major cause of the decreased hepatic SAM concentration (Hoffman 1984), since provision of a

dietary choline source likely prevented any ethionine-induced deficit in choline biosynthesis (Farber 1963).

Dietary SBTI appeared to increase the susceptibility of the rats to the effects of ethionine. Lowest levels of SAM and lower SAM/SAH ratios were measured in rats fed SBTI combined with DL-ethionine, especially at the 0.05% dietary ethionine level. The lack of difference in hepatic SAM with increasing dietary SBTI, consistently seen at the highest level of ethionine fed, may have resulted from an increase in SAM accumulation because of the higher ethionine level. Hyde and Poirier (1982) suggested that higher hepatic SAM measured in rats fed 0.3 compared to 0.1% DL-ethionine was consistent with greater inhibition of transmethylation and SAM accumulation with the higher SAE levels in rats fed the higher level of ethionine.

The ratio of hepatic SAE/SAM is also an indicator of availability of methyl groups with ethionine intoxication. The higher ratios of SAE/SAM with increased dietary ethionine detected in the first experiment, were indicative of decreased availability of activated methionine for transmethylation reactions and support similar conclusions based on lower hepatic SAM and decreased SAM/SAH ratios.

In earlier studies (Section III. 2), it was found that the 0.3% dietary level of ethionine was not well tolerated by weanling rats, especially in combination with SBTI. This intolerance may have resulted from an interaction of three factors: limited methionine availability and supply with high dietary SBTI; ethionine competition with methionine activation leading to limited SAM formation and reduced methylation reactions; and the high methionine requirements of the young growing rats. In the current study, the 0.05 and 0.10% levels of dietary ethionine were better tolerated with no early morbidity. It seems likely, however, that

higher sulfur amino acid requirements of the younger animals continued to be a factor since SAM concentrations and SAM/SAH ratios were lowest in the first month of the experiment.

Despite the demonstrated effects of SBTI on the transmethylation-transsulfuration pathway and the known influence of both lipotrope deficit and ethionine on development of fatty liver (Farber 1967), hepatic lipid levels decreased with increasing dietary SBTI and were lower when SBTI and ethionine were fed compared to ethionine alone (Table 5.6). This result contrasts to findings of Henning et al. (1989) where higher liver lipid was measured in methionine and choline deficient rats, deprived of folate or with induced enhancement of methyl group utilization (Henning et al. 1989).

The hypolipidemic influence of the soy protein and fibre used in the current study likely contributed to these differences. It has been reported that these can affect both lipid absorption, metabolism and hepatic deposition (Hamilton and Carroll 1976, Carroll 1983). The presence of SBTI may also have contributed to this effect since it has been shown that both raw soy meal and isolated trypsin inhibitors decreased fat absorption and enhanced secretion of cholesterol, fatty acids and other bile components (Rackis 1974).

In the second experiment, equivalent hepatic levels of SAM and ratios of SAM/SAH in rats fed the casein-based diet or the two soy diets without ethionine, may have resulted from the provision of choline and theoretically adequate methionine in each diet. Each diet was suitably supplemented with crystalline L-amino acids to meet rat growth requirements based on amino acid analysis of the protein sources used (Appendix A).

As seen in the first experiment, dietary ethionine in combination with SBTI resulted in lower hepatic SAM at 4 weeks. The failure to measure significantly lower SAM at 2 weeks in SBTI + ethionine-fed rats, compared to levels in rats fed the two SBTI diets without ethionine, may reflect the provision of choline and methionine in the diets. Time may be

required for depletion of body stores of sulfur amino acids to a level sufficient to affect SBTI + ethionine induced changes in the transsulfuration pathway, especially when dietary choline and methionine are not limiting. Alternatively, as suggested for the first experiment, the higher SAM concentrations may have resulted from impairment of transmethylation and SAM accumulation because of the effects of the dietary ethionine. It is to be noted that hepatic SAM concentrations of rats fed the 0.2% ethionine-containing diets were consistently higher than those of rats fed at the lower ethionine levels of the previous experiment.

The greater rise in hepatic SAM that occurred in rats fed the ethionine-containing diet with the higher level of SBTI may have resulted from competing mechanisms. Supplementary methionine may act to negate the drop in SAM biosynthesis by partially overcoming competition by dietary ethionine. The highly available, crystalline L-methionine supplement used could readily counteract the effects of ethionine on methionine activation. At the same time the increased methionine may not have been effective at overcoming SAE and ethionine-induced inhibition of later steps in the transsulfuration pathway. As a result SAM would tend to accumulate.

The absence of increased hepatic SAM concentrations with L-cysteine supplementation of the SBTI + ethionine-containing diets (most evident at 4 weeks) also likely involved several factors. It has been proposed that cysteine-rich SBTI are not only refractory to digestion but also increase the synthesis and loss of cysteine-rich proteinases by formation and loss of inhibitor-enzyme complexes (Kakade et al. 1969). Cysteine supplementation could enhance the pancreatic trophic response by supplying more of the limiting cysteine required for pancreatic proteinase synthesis (Gumbmann and Friedman 1987). Blockage of transmethylation reactions by high levels of ethionine and its SAE metabolite, may have negated any potential sparing effect of cysteine on methionine by inhibition of the

transsulfuration pathway (Finkelstein and Mudd 1967). Finkelstein et al. (1986) also reported that cystine-induced reduction of transsulfuration and increased methionine retention in rats were dependent upon relatively high cystine levels and marginal methionine status of the basal diet used.

The higher concentrations of hepatic SAE measured in rats of the second experiment compared to those of the first experiment , were expected because of the higher dietary level of ethionine used. In most cases supplementary methionine appeared to lower hepatic SAE compared to levels in rats fed ethionine alone, although this was significant only at the highest level of SBTI at 4 weeks. Lower SAE levels with methionine supplementation and lack of change with cysteine supplementation would be consistent with increased methionine supply competing with ethionine for activation in the initial step of the transmethylation pathway. Failure of cysteine to cause a similar lowering remains to be explained.

The drop in hepatic SAM/SAH ratio and the high SAE/SAM ratio with intake of dietary SBTI and high ethionine were consistent with reduced availability of methyl groups, as seen in the first experiment. Provision of supplementary methionine increased the SAM/SAH ratio and decreased the SAE/SAM ratio indicating that methionine was effective at overcoming the decreased methyl donor availability. The ineffectiveness of supplementary cysteine supplementation at improving these ratios may have implications in terms of selection of suitable sulfur amino acid supplements required in infant formulas, especially those that are soy based. Any potentially desired amelioration of transmethylation transsulfuration parameters would have to be balanced against potential absolute requirements for a cysteine component to the dietary sulfur amino acid supply for infants (Atkinson and Hanning 1989).

Differences in hepatic lipid levels were much less evident in the second experiment. The shorter duration of the second experiment was likely a factor. In this experiment,

ethionine was always fed in combination with SBTI. It would appear that at both 2 weeks and 4 weeks, the presence of SBTI even at the low level used was sufficient to prevent significant ethionine-induced accumulation of hepatic lipid. A similar protective effect of SBTI on ethionine-induced fatty liver was noted in the first experiment.

Changes in concentrations of liver free amino acids measured in rats of the second experiment, reflect changes in the transmethylation-transsulfuration pathway already noted in terms of S-adenosyl derivatives. The equivalent levels of key essential amino acids such as methionine, threonine and lysine in both the casein and soy-based diets without ethionine support the adequacy of these diets for rat growth. The presence of dietary ethionine resulted in a significant increase in liver free methionine and lysine at 4 weeks, suggesting decreased utilization of these amino acids in protein synthesis. Utilization of ATP in SAE formation may have limited the availability of this compound both for methionine activation in transmethylation reactions and for general amino acid activation required in protein synthesis (Bhagavan 1978). It is noted that both methionine and cysteine supplementation at the higher level of dietary SBTI were effective at moderating high levels of liver methionine, suggesting that utilization or availability of both amino acids had been affected by SBTI.

Changes in levels of liver free non-essential amino acids that are substrates, precursors or products of the transsulfuration pathway, also indicate decreased transsulfuration with intake of dietary SBTI and ethionine. Serine, a co-substrate for cystathionine synthesis (Finkelstein 1990), increased in response to increasing SBTI at both 2 and 4 weeks, suggesting its accumulation and decreased transsulfuration. Accumulation of the serine precursor glycine, in response to ethionine similarly suggests decreased serine utilization via the transsulfuration pathway. Accumulation of glycine may also indicate decreased SAM availability for sarcosine formation (Finkelstein 1990).

The lowering of liver free serine and glycine levels with methionine supplementation of the SBTI + ethionine-containing diets, are consistent with their increased utilization and input into transsulfuration. The high level of liver free taurine measured in rats fed the SBTI + ethionine containing diets may reflect a toxic effect of ethionine on liver lipid and bile acid transport. No rise in taurine (a cysteine-derived end product of transsulfuration), was seen in rats fed diets containing SBTI without ethionine. The moderation of taurine levels with methionine supplementation, suggest alleviation of this toxic effect, likely via methionine influence on liver SAE (Brada et al. 1976). Increased availability of cysteine precursor explains the higher liver free taurine levels occurring with cysteine supplementation.

It is concluded that dietary SBTI can induce a methyl group deficit as indicated by changes in S-adenosyl derivatives and amino acids related to the transmethylation-transsulfuration pathway of methionine metabolism. In addition, SBTI appear to increase the severity of methyl group deficit and changes to the transmethylation-transsulfuration pathway induced by the hepatocarcinogen ethionine. The continued presence of these changes for up to 3 months suggests that they may be a key factor in the antinutritional and carcinogenic influences of SBTI.

The need to consider methionine/cysteine ratios in the formulation and sulfur amino acid supplementation of infant formulas is underlined by the varying effectiveness of the two amino acids in normalizing changes in transmethylation-transsulfuration parameters induced by SBTI and DL-ethionine.

TABLE 5.1 Hepatic s-adenosyl-derivative concentrations[1,2] in male rats fed SBTI[3] and/or DL-ethionine for 4 weeks.

	Diet								
	1	2	3	4	5	6	7	8	9
SBTI[3]	0	448	808	0	448	808	0	448	808
DL-ethionine (%)	0	0	0	0.05	0.05	0.05	0.10	0.10	0.10
S-adenosylhomocysteine	9.5±0.6^{c}	6.8±0.4^{ab}	8.3±0.8^{bc}	7.9±0.9^{abc}	8.3±1.0^{bc}	6.7±0.6^{ab}	7.5±0.6^{abc}	6.0±0.2^{a}	7.7±0.4^{abc}
S-adenosylmethionine	41.3±4.5^{c}	22.5±1.8^{b}	20.6±1.9^{b}	21.8±2.6^{b}	12.5±2.0^{a}	8.8±0.6^{a}	11.7±1.0^{a}	12.2±0.9^{a}	9.0±0.5^{a}
S-adenosylethionine	0^{a}	0^{a}	0^{a}	125±10^{b}	122±13^{b}	122±9^{b}	297±21^{c}	304±18^{c}	321±20^{c}
S-adenosylmethionine / S-adenosylhomocysteine	4.4±0.4^{d}	3.4±0.4^{c}	2.7±0.5^{bc}	2.8±0.3^{bc}	1.7±0.3^{a}	1.4±0.2^{a}	1.6±0.1^{a}	2.0±0.2^{ab}	1.2±0.1^{a}
S-adenosylethionine / S-adenosylmethionine	0^{a}	0^{a}	0^{a}	6.1±0.6^{b}	11.2±2.0^{bc}	14.0±0.8^{c}	27.4±4.3^{d}	26.1±3.3^{d}	35.7±1.5^{e}

[1]nmol/g liver, means ± SEM for 8 animals per group.
[2]Means with different superscripts in the same row differ significantly ($P<0.05$).
[3]SBTI: Soybean trypsin inhibitor measured as mg trypsin inhibited per 100 g diet.

TABLE 5.2 Hepatic s-adenosyl-derivative concentrations[1,2] in male rats fed SBTI[3] and/or DL-ethionine for 8 weeks.

	Diet								
	1	2	3	4	5	6	7	8	9
SBTI[3]	0	448	808	0	448	808	0	448	808
DL-ethionine (%)	0	0	0	0.05	0.05	0.05	0.10	0.10	0.10
S-adenosylhomocysteine	7.0±0.6^{b}	6.7±0.3^{ab}	5.2±0.2^{a}	6 9±0.7^{b}	6.4±0.7^{ab}	5.1±0.4^{a}	6.5±0.7^{ab}	5.0±0.4^{a}	5.7±0.6^{ab}
S-adenosylmethionine	41.9±2.6^{d}	26.6±0.6^{bc}	30 1±1.6^{c}	23.8±1.9^{b}	15.3±1.1^{a}	15.7±1.1^{a}	13.2±0.7^{a}	16.8±3.1^{a}	13.2±0.9^{a}
S-adenosylethionine	0^{a}	0^{a}	0^{a}	88±6^{b}	95±8^{b}	102±8^{b}	172±10^{c}	238±23^{d}	236±21^{d}
S-adenosylmethionine / S-adenosylhomocysteine	6.7±0.7^{e}	5.1±0.7^{d}	5.9±0.4^{de}	3.9±0.4^{c}	2.5±0.1^{ab}	3.2±0.2^{abc}	2.1±0.2^{a}	3.4±0 4^{bc}	2.4±0.2^{ab}
S-adenosylethionine / S-adenosylmethionine	0^{a}	0^{a}	0^{a}	3.9±0.4^{b}	6.3±0.6^{b}	6.8±0.7^{b}	13.5±1.6^{c}	14.5±1.2^{c}	18.5±2.0^{d}

[1]nmol/g liver, means ± SEM for 8 animals per group.
[2]Means with different superscripts in the same row differ significantly ($P<0.05$).
[3]SBTI: Soybean trypsin inhibitor measured as mg trypsin inhibited per 100 g diet.

TABLE 5.3 Hepatic s-adenosyl-derivative concentrations[1,2] in male rats fed SBTI[3] and/or DL-ethionine for 12 weeks.

	Diet								
	1	2	3	4	5	6	7	8	9
SBTI[3]	0	448	808	0	448	808	0	448	808
DL-ethionine (%)	0	0	0	0.05	0 05	0 05	0.10	0.10	0.10
S-adenosylhomocysteine	8.3±0 8^{c}	6.6±0.3^{ab}	7.2±0.4^{bc}	5.7±0 5^{ab}	5 5±0 5^{a}	7.2±0.4^{bc}	6 9±0.6^{abc}	5 3±0.3^{a}	6.0±0.6^{ab}
S-adenosylmethionine	46 7±4.0^{c}	31.7±2.4^{b}	30.8±1 8^{b}	17 3±1 1^{a}	15 8±1 2^{a}	15.9±1.2^{a}	15.5±1.0^{a}	14.4±0.6^{a}	14.3±0.8^{a}
S-adenosylethionine	0^{a}	0^{a}	0^{a}	80±8^{b}	80±6^{b}	91±7^{b}	190±26^{c}	216±23^{c}	218±13^{c}
S-adenosylmethionine / S-adenosylhomocysteine	5 7±0.3^{d}	4.9±0.4^{cd}	4.4±0.5^{c}	3 2±0.3^{b}	3.0±0 2^{ab}	2.2±0.2^{a}	2.4±0 3^{ab}	2.7±0.1^{ab}	2.7±0.3^{ab}
S-adenosylethionine / S-adenosylmethionine	0^{a}	0^{a}	0^{a}	4.6±0 3^{b}	5 2±0 6^{b}	5.9±0 5^{b}	12.5±1.8^{c}	15.5±2.0^{d}	15.1±1.0^{cd}

[1]nmol/g liver, means ± SEM for 8 animals per group
[2]Means with different superscripts in the same row differ significantly ($P<0.05$).
[3]SBTI. Soybean trypsin inhibitor measured as mg trypsin inhibited per 100 g diet

TABLE 5.4 Hepatic s-adenosyl-derivative concentrations[1,2] in male rats fed SBTI[3] and/or DL-ethionine with and without supplementary sulfur amino acids for 2 weeks.

	Diet								
	1	2	3	4	5	6	7	8	9
SBTI[3]	0	421	421	421	421	775	775	775	775
DL-ethionine (%)	0	0	0.2	0.2	0.2	0	0.2	0.2	0.2
SAA supplement[4]	0	0	0	M	C	0	0	M	C
S-adenosylhomocysteine	10.0±0.2	9.8±0.4	9.5±0.8	8.7±0.5	10.2±1.0	8.7±0.6	8.5±0.8	9.0±0.2	9.1±0.7
S-adenosylmethionine	37.7±2.8^{ab}	44.7±1.8^{ab}	33.3±4.1^{ab}	47.5±7.7^{b}	33.9±4.2^{ab}	42.9±2.3^{ab}	30.0±5.0^{a}	74.3±7.1^{c}	39±4.7^{ab}
S-adenosylethionine	0^{a}	0^{a}	797±47^{cd}	691±39^{bc}	876±58^{d}	0^{a}	626±42^{b}	671±55^{b}	737±56^{bc}
S-adenosylmethionine / S-adenosylhomocysteine	3.8±0.2^{a}	4.7±0.3^{a}	3.6±0.4^{a}	5.3±0.9^{a}	3.5±0.5^{a}	5.0±0.3^{a}	3.6±0.6^{a}	8.3±0.7^{b}	3.9±0.4^{a}
S-adenosylethionine / S-adenosylmethionine	0^{a}	0^{a}	26.2±2.9^{de}	18.3±3.7^{c}	27.7±2.8^{e}	0^{a}	23.7±2.7^{cde}	9.5±1.2^{b}	20.4±2.2^{cd}

[1]nmol/g liver, means ± SEM for 8 animals per group.
[2]Means with different superscripts in the same row differ significantly ($P<0.05$)
[3]SBTI: Soybean trypsin inhibitor measured as mg trypsin inhibited per 100 g diet.
[4]SAA supplement: M, L-methionine (3.35 mmol/100 g diet); C, L-cysteine (3.35 mmol/100 g diet)

TABLE 5.5 Hepatic S-adenosyl-derivative concentrations[1,2] in male rats fed SBTI[3] and/or DL-ethionine with and without supplementary sulfur amino acids for 4 weeks.

	Diet								
Dietary:	1	2	3	4	5	6	7	8	9
SBTI[3]	0	421	421	421	421	775	775	775	775
DL-ethionine (%)	0	0	0.2	0.2	0.2	0	0.2	0.2	0.2
SAA supplement[4]	0	0	0	M	C	0	0	M	C
S-adenosylhomocysteine	8.9±0.7	8.7±0.6	7.7±0.5	8.4±0.8	6.9±0.6	8.7±1.0	7.6±0.6	7.4±0.4	8.9±1.5
S-adenosylmethionine	44.5±2.9^{bc}	42.2±2.8^{b}	26.1±2.1^{a}	41.6±4.0^{b}	24.5±1.7^{a}	45.6±3.9^{bc}	20.6±1.4^{a}	55.0±7.7^{c}	23.3±2.5^{a}
S-adenosylethionine	0^{a}	0^{a}	682±39^{cd}	596±44^{bc}	662±24^{bcd}	0^{a}	772±77^{d}	562±30^{bc}	716±45^{d}
S-adenosylmethionine / S-adenosylhomocysteine	5.1±0.3^{bcd}	4.9±0.2^{bcd}	3.3±0.2^{ab}	5.3±0.7^{cd}	3.5±0.3^{abc}	5.9±1.0^{de}	2.9±0.4^{a}	7.3±0.7^{e}	3.1±0.5^{a}
S-adenosylethionine / S-adenosylmethionine	0^{a}	0^{a}	27.8±3.4^{c}	14.9±1.6^{b}	27.7±1.8^{c}	0^{a}	37.7±2.9^{d}	11.1±1.2^{b}	32.4±2.6^{cd}

[1]nmol/g liver, means ± SEM for 8 animals per group.
[2]Means with different superscripts in the same row differ significantly ($P<0.05$).
[3]SBTI: Soybean trypsin inhibitor measured as mg trypsin inhibited per 100 g diet.
[4]SAA supplement: M, L-methionine (3.35 mmol/100 g diet); C, L-cysteine (3.35 mmol/100 g diet).

TABLE 5.6 Hepatic lipid content[1,2] of male rats fed SBTI[3] and/or DL-ethionine over 12 weeks

	Diet								
Dietary:	1	2	3	4	5	6	7	8	9
SBTI[3]	0	448	808	0	448	808	0	448	808
DL-ethionine (%)	0	0	0	0.05	0.05	0.05	0.10	0.10	0.10
Week 4	93±7^{cd}	82±5^{abc}	69±4^{ab}	140±9^{e}	105±5^{d}	93±4^{cd}	88±9^{bcd}	75±4^{abc}	67±6^{a}
Week 8	105±9^{abc}	84±5^{ab}	78±5^{a}	169±14^{e}	120±7^{cd}	122±9^{cd}	135±17^{d}	112±7^{bcd}	88±8^{ab}
Week 12	130±12^{cd}	93±5^{ab}	80±9^{a}	183±14^{e}	144±10^{d}	124±13^{bcd}	180±21^{e}	101±8^{abc}	96±5^{abc}

[1]mg/g liver, means ± SEM for 8 animals per group.
[2]Means with different superscripts in the same row differ significantly ($P < 0.05$).
[3]SBTI: soybean trypsin inhibitor measured as mg trypsin inhibited per 100 g diet.

TABLE 5.7 Hepatic lipid content[1,2] of male rats fed SBTI[3] and DL-ethionine with and without supplementary sulfur amino acids over 4 weeks.

	Diet								
Dietary:	1	2	3	4	5	6	7	8	9
SBTI[3]	0	421	421	421	421	775	775	775	775
DL-ethionine (%)	0	0	0.2	0.2	0.2	0	0.2	0.2	0.2
SAA Supplement[4]	0	0	0	M	C	0	0	M	C
Week 2	69 ± 4^{ab}	69 ± 1^{ab}	65 ± 8^{a}	69 ± 5^{ab}	71 ± 4^{bc}	66 ± 2^{a}	68 ± 3^{ab}	75 ± 3^{c}	68 ± 3^{ab}
Week 4	81 ± 2^{e}	77 ± 2^{de}	64 ± 1^{a}	77 ± 3^{de}	68 ± 1^{ab}	73 ± 7^{cd}	71 ± 3^{bc}	73 ± 4^{cd}	71 ± 1^{bc}

[1]mg/g liver, means ± SEM of 4 pooled samples per treatment, each pooled sample representing 2 rats.
[2]Means with different superscripts in the same row differ significantly, ($P < 0.05$).
[3]SBTI: soybean trypsin inhibitor measured as mg trypsin inhibited per 100 g diet.
[4]SAA supplement: M, L-methionine (3.35 mmol/100 g diet); C, L-cysteine (3.35 mmol/100 g diet).

TABLE 5.8 Effects of supplementary sulfur amino acids on concentrations[1,2] of selected free amino acids in livers of rats fed SBTI[2] and/or DL-ethionine for 2 weeks.

	Diet									
Dietary:	1	2	3	4	5	6	7	8	9	SEM[4]
SBTI[3]	0	421	421	421	421	775	775	775	775	
DL-ethionine(%)	0	0	0.2	0.2	0.2	0	0.2	0.2	0.2	
SAA supplement[5]	0	0	0	M	C	0	0	M	C	
L-Met	0.38	0.44	0.54	0.59	0.52	0.35	0.60	0.42	0.49	0.07
L-Thr	4.3^{bcd}	4.8^{cd}	3.9^{abc}	2.0^{a}	2.7^{ab}	5.9^{d}	3.4^{abc}	2.4^{ab}	3.8^{abc}	0.6
L-Lys	2.5^{ab}	2.1^{a}	2.5^{ab}	3.6^{b}	2.9^{ab}	2.0^{a}	3.6^{b}	2.8^{ab}	2.8^{ab}	0.4
L-Ser	2.9^{abc}	3.8^{bc}	4.7^{cd}	1.8^{a}	3.5^{abc}	5.6^{d}	2.4^{abc}	2.1^{ab}	3.9^{bc}	0.6
L-Gly	5.6^{a}	7.6^{a}	13.5^{b}	6.7^{a}	14.5^{b}	7.5^{a}	15.7^{b}	5.9^{a}	16.0^{b}	1.0
L-Ala	6.6^{a}	6.7^{a}	8.0^{a}	6.6^{a}	7.1^{a}	6.5^{a}	11.3^{b}	6.6^{a}	7.8^{a}	0.9
L-Tau	1.8^{a}	1.5^{a}	11.0^{bc}	9.6^{b}	16.4^{c}	1.1^{a}	9.0^{b}	7.6^{b}	12.8^{bc}	2.0

[1]nmoles per mg freeze-dried tissue; means ± SEM of 4 animals per group.
[2]Means with different superscripts in the same row differ significantly ($P < 0.05$).
[3]SBTI: Soybean trypsin inhibitor measured as mg trypsin inhibited per 100 g diet.
[4]SEM calculated from analysis of variance data.
[5]SAA supplement: M, L-methionine (3.35 mmol/100 g diet); C, L-cysteine (3.35 mmol/100 g diet).

TABLE 5.9 Effects of supplementary sulfur amino acids on concentrations[1,2] of selected free amino acids in livers of rats fed SBTI[2] and/or DL-ethionine for 4 weeks.

	Diet									
Dietary:	1	2	3	4	5	6	7	8	9	SEM[4]
SBTI[3]	0	421	421	421	421	775	775	775	775	
DL-ethionine(%)	0	0	0.2	0.2	0.2	0	0.2	0 2	0.2	
SAA supplement[5]	0	0	0	M	C	0	0	M	C	
L-Met	0.34[a]	0.36[a]	0.58[cd]	0.46[abc]	0.54[bcd]	0.35[a]	0.64[d]	0.42[ab]	0.36[a]	0.04
L-Thr	3.6[b]	3.3[ab]	3.2[ab]	2.7[ab]	3.3[b]	4.1[b]	3.6[b]	1.8[a]	6.2[c]	0.5
L-Lys	1.8[a]	1.5[a]	4.3[d]	2.8[b]	4.5[d]	1.6[a]	3.8[cd]	3.1[bc]	4.3[d]	0.3
L-Ser	3.7[ab]	6.0[cd]	4.6[bc]	2.6[a]	5.1[bcd]	6.8[d]	4.8[bc]	2.7[a]	5.4[bcd]	0.6
L-Gly	6.9[a]	7.9[a]	14.9[c]	9.5[ab]	15.0[c]	6.8[a]	19.9[d]	8.4[a]	12.8[bc]	1.4
L-Ala	6.9[ab]	7.8[abc]	9.9[bc]	6 8[ab]	10.6[c]	7.8[abc]	8.5[abc]	5.9[a]	9.7[bc]	1.0
L-Tau	2 6[a]	2.7[a]	13.2[c]	10.2[bc]	22.2[d]	1.7[a]	12.3[c]	5.5[ab]	20.0[d]	2.1

[1]nmoles per mg freeze-dried tissue; means ± SEM of 4 animals per group.
[2]Means with different superscripts in the same row differ significantly ($P < 0.05$).
[3]SBTI: Soybean trypsin inhibitor measured as mg trypsin inhibited per 100 g diet.
[4]SEM calculated from analysis of variance data.
[5]SAA supplement: M, L-methionine (3.35 mmol/100 g diet); C, L-cysteine (3.35 mmol/100 g diet).

Figure 5.1 Elution profiles of S-adenosyl-derivatives of methionine and ethionine in standard (std) solution and in representative liver extract (rec11a) obtained by SCX-cation exchange HPLC chromatography.

ADE: adenosine

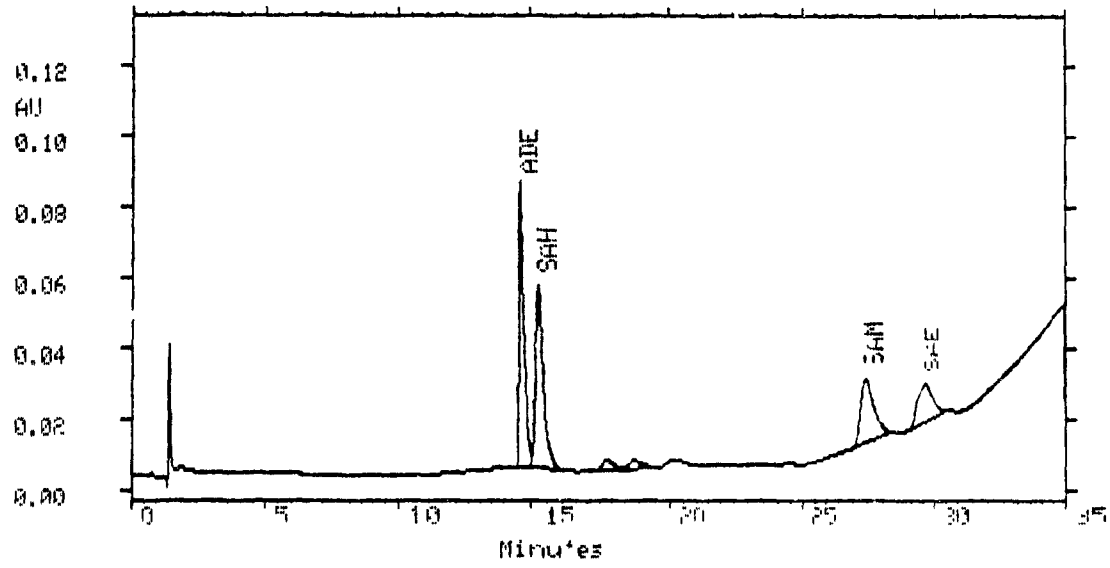
SAH: S-adenosylhomocysteine

SAM: S-adenosylmethionine

SAE: S-adenosylethionine

Figure 5.1 Elution profile of S-adenosyl derivatives by SCX-cation exchange

Chromatogram of std



Chromatogram of recl1a

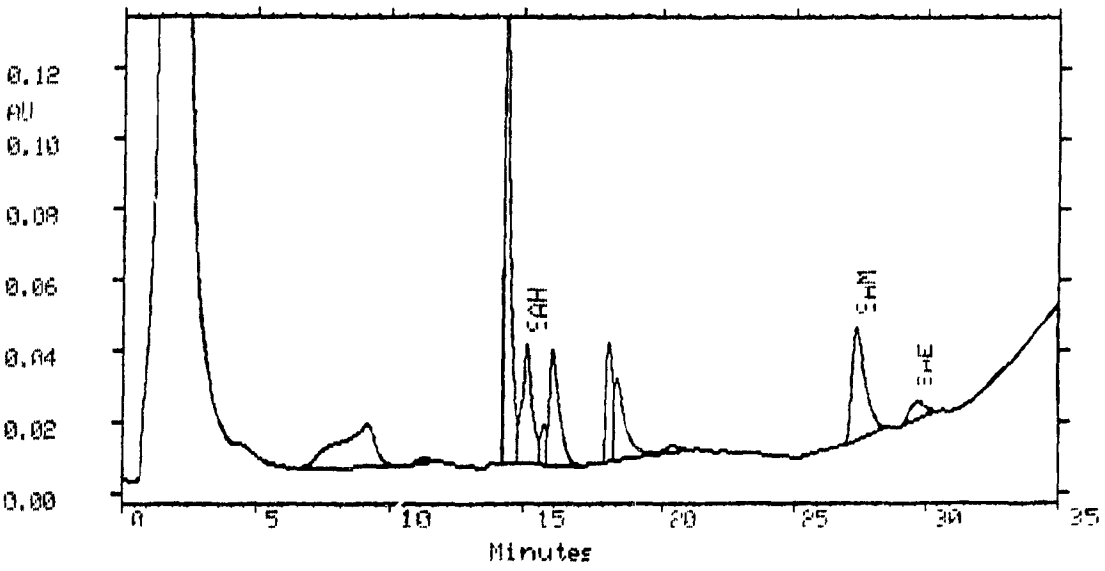


Figure 5.2 Liver S-adenosylmethionine concentrations [SAM] (nM/g liver tissue, mean ± SEM of 8 rats per treatment) in rats fed combinations of soybean trypsin inhibitors (SBTI: 0, 448 or 808 mg trypsin inhibitor/100g diet) and DL-ethionine (0, 0.05 or 0.10% of diet) over 4, 8 and 12 weeks.

Bars representing treatment means, with different letters at same week, differ significantly ($P < 0.05$).

Figure 5.2

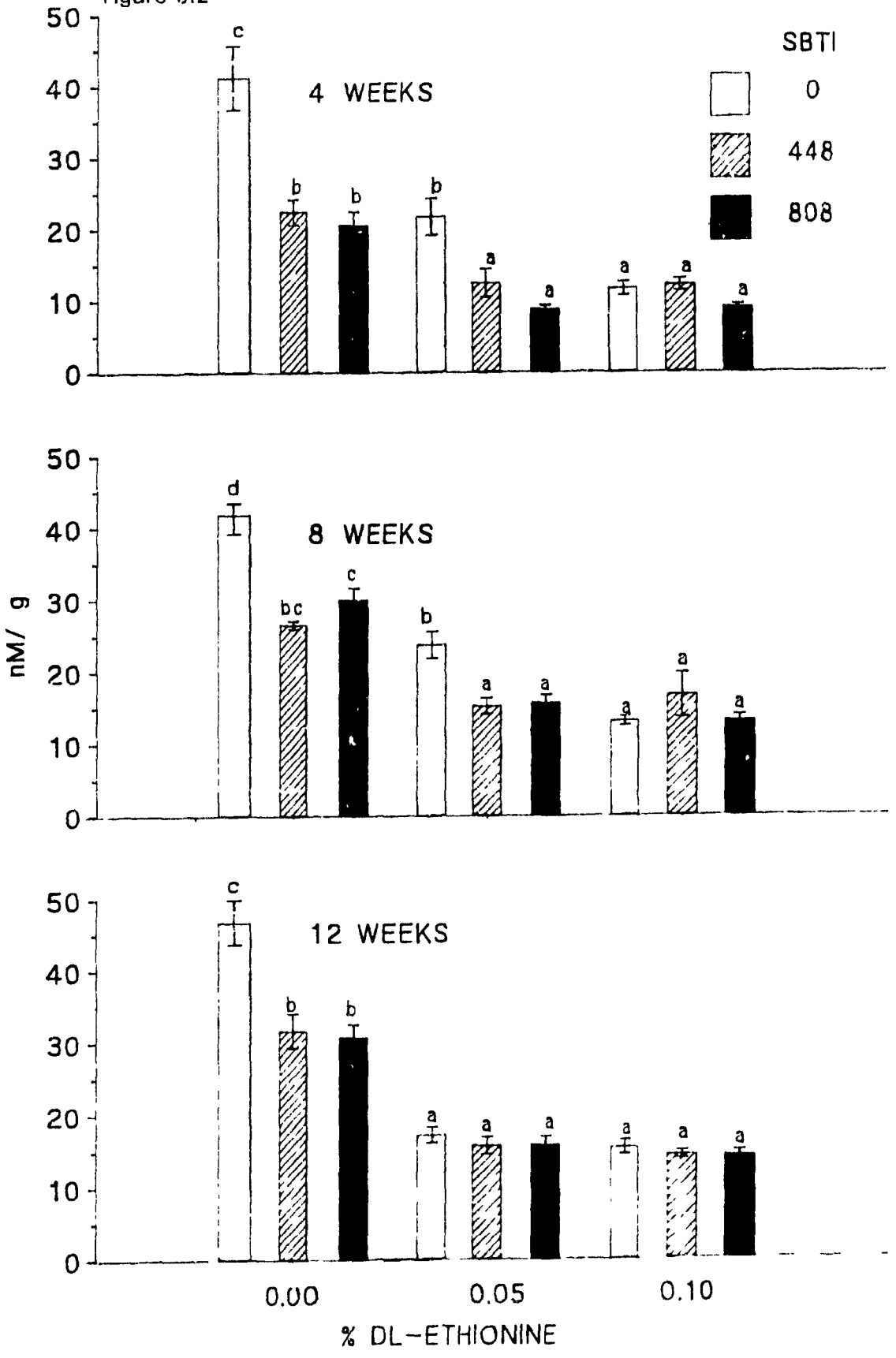

LIVER [SAM]
4 WEEKS
8 WEEKS
12 WEEKS
SBTI
0
448
808
nM/ g
% DL-ETHIONINE
0.00
0.05
0.10

Figure 5.3 Liver S-adenosylmethionine/S-adenosylhomocysteine ratios (SAM/SAH, mean ± SEM of 8 rats per treatment) in rats fed combinations of soybean trypsin inhibitors (SBTI :0, 448 or 808 mg trypsin inhibitor/100g diet) and DL-ethionine (0, 0.05 or 0.10% of diet) over 4, 8 and 12 weeks.

Bars representing treatment means, with different letters at same week, differ significantly ($P<0.05$).

LIVER [SAM/SAH]

Figure 5.3

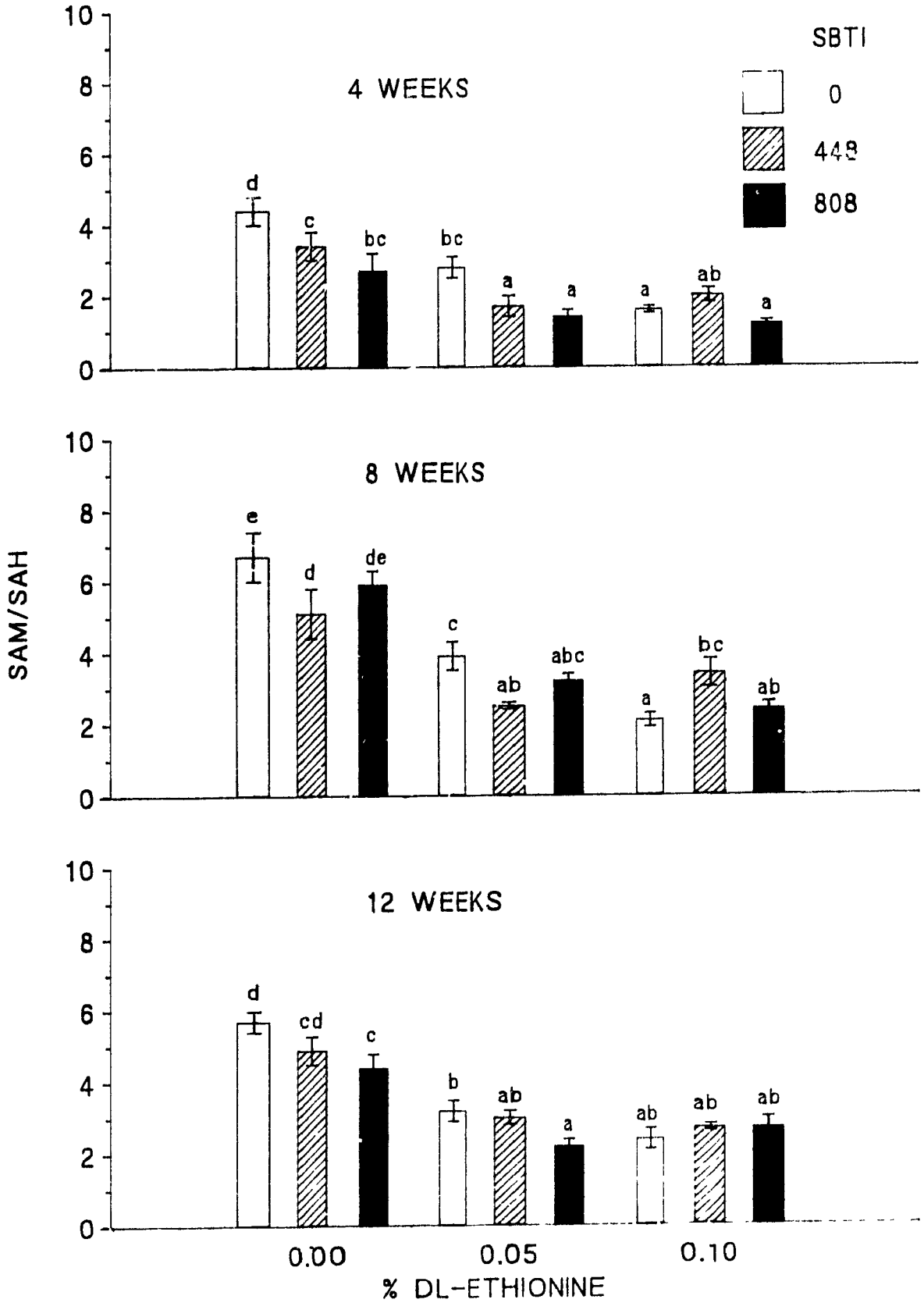


Figure 5.4 Liver S-adeonosylethionine/S-adenosylmethionine ratios (SAE/SAM, mean ± SEM for 8 rats per treatment) in rats fed combinations of soybean trypsin inhibitors (SBTI; 0, 448 or 808 mg trypsin inhibitor/100g diet) and DL-ethionine (0, 0.05 or 0.10% of diet) over 4, 8 and 12 weeks.

Bars representing treatment means with different letters at same week, differ significantly ($P < 0.05$).

Figure 5.4

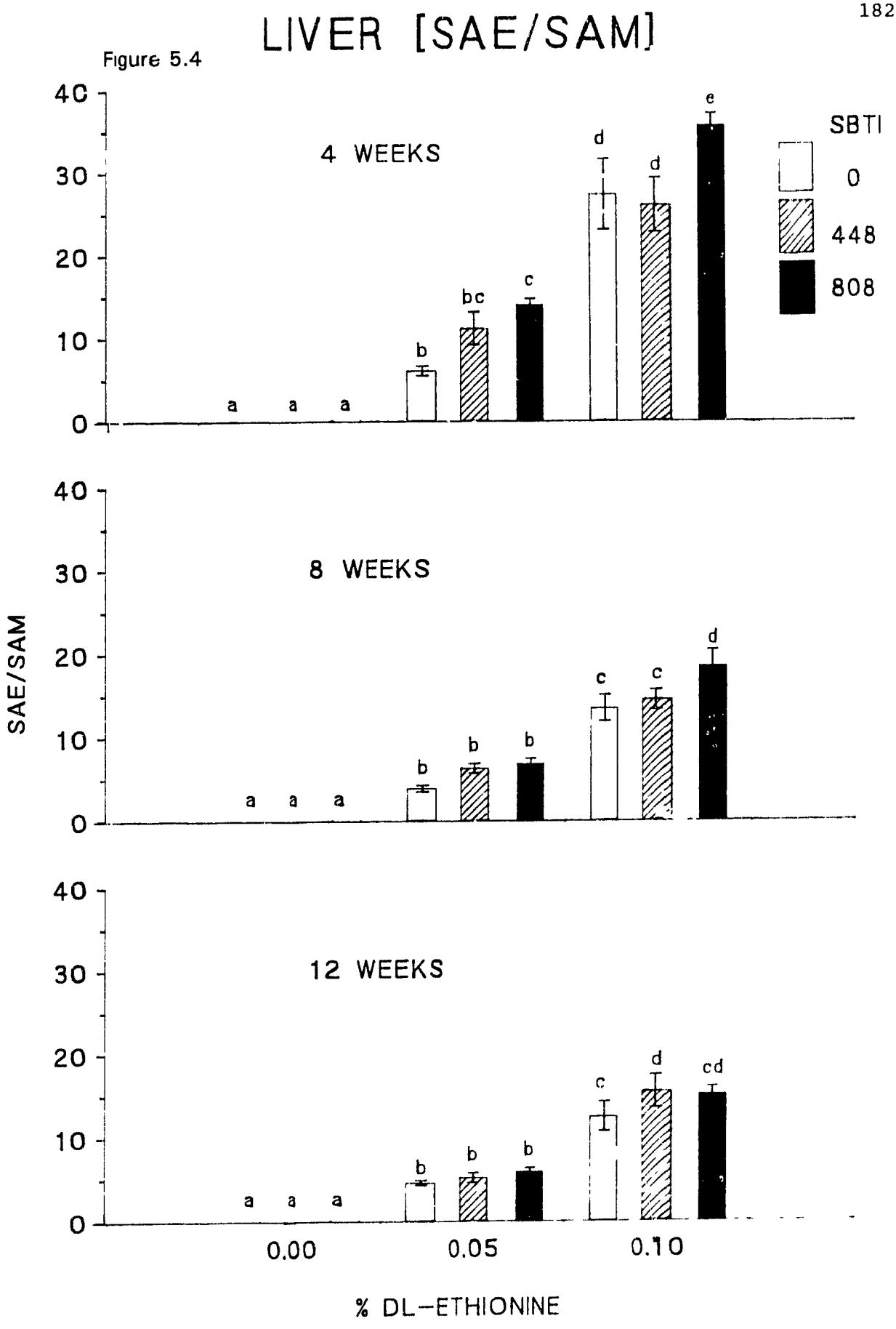

LIVER [SAE/SAM]
4 WEEKS
8 WEEKS
12 WEEKS
SAE/SAM
SBTI
0
448
808
0.00
0.05
0.10
% DL-ETHIONINE

Figure 5.5 Liver S-adenosylmethionine concentrations [SAM] (nM/g tissue) in rats fed diets differing in trypsin inhibitor content and presence or absence of supplementary sulfur amino acids over 2 and 4 weeks.

Bars representing treatment means with different letters at same week, differ significantly ($P < 0.05$).

Diet Key

cas: Casein control (0 trypsin inhibitor) (Diet 1)

ti : Low SBTI diet
(421 mg trypsin inhibited/100 g diet) (Diet 2)

tiE: Low SBTI diet + 0.2% DL-ethionine (Diet 3)

tiEM: Low SBTI diet + 0.2% DL-ethionine
+ L-methionine 3.35 mM/100 g diet) (Diet 4)

tiEC: Low SBTI diet + 0.2% DL-ethionine
+ L-cysteine (3.35 mM/100g diet) (Diet 5)

TI: High SBTI diet (775 mg trypsin inhibited/100g diet) (Diet 6)

TIE: High SBTI diet + 0.2% DL-ethionine (Diet 7)

TIEM: High SBTI diet + 0.2% DL-ethionine
+ L-methionine (3.35 mM/100g diet) (Diet 8)

TIEC: High SBTI diet + 0.2% DL-ethionine
+ L-cysteine (3.35 mM/100 g diet) (Diet 9)

Figure 5.5

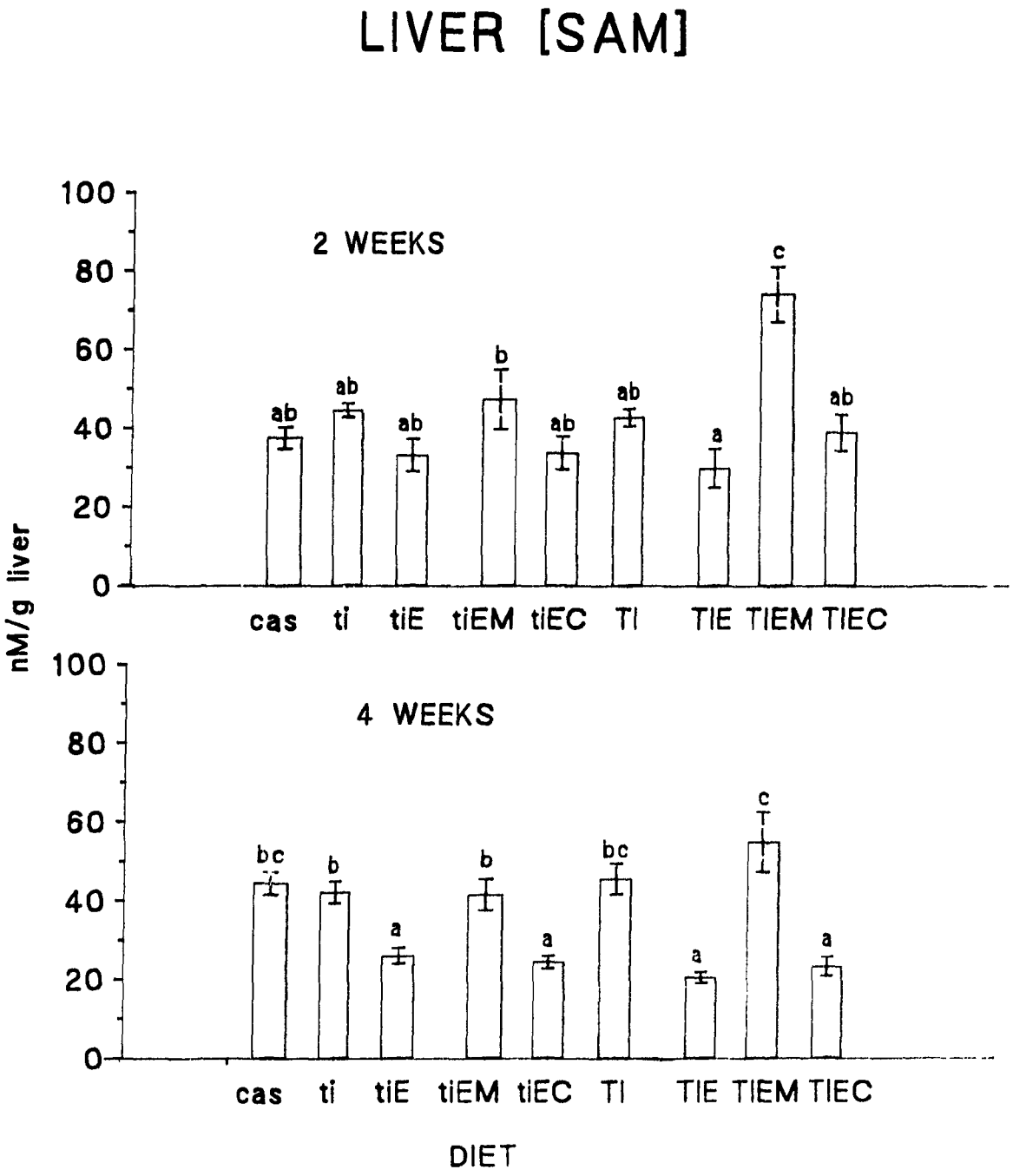

LIVER [SAM]
2 WEEKS
4 WEEKS
nM/g liver
100
80
60
40
20
0
ab
ab
ab
b
ab
ab
a
c
ab
bc
b
a
b
a
bc
a
c
a
cas
ti
tiE
tiEM
tiEC
TI
TIE
TIEM
TIEC
DIET

Figure 5.6 Liver S-adenosylmethionine/S-adenosylhomocysteine ratios in rats fed diets differing in trypsin inhibitor content and presence or absence of supplementary sulfur amino acids over 2 and 4 weeks.

Bars representing treatment means with different letters at the same week, differ significantly ($P<0.05$).

Diet Key

cas: Casein control (0 trypsin inhibitor) (Diet 1)

ti : Low SBTI diet
(421 mg trypsin inhibited/100 g diet) (Diet 2)

tiE: Low SBTI diet + 0.2% DL-ethionine (Diet 3)

tiEM: Low SBTI diet + 0.2% DL-ethionine
+ L-methionine 3.35 mM/100 g diet) (Diet 4)

tiEC: Low SBTI diet + 0.2% DL-ethionine
+ L-cysteine (3.35 mM/100g diet) (Diet 5)

TI: High SBTI diet (775 mg trypsin inhibited/100g diet) (Diet 6)

TIE: High SBTI diet + 0.2% DL-ethionine (Diet 7)

TIEM: High SBTI diet + 0.2% DL-ethionine
+ L-methionine (3.35 mM/100g diet) (Diet 8)

TIEC: High SBTI diet + 0.2% DL-ethionine
+ L-cysteine (3.35 mM/100 g diet) (Diet 9)

Figure 5.6

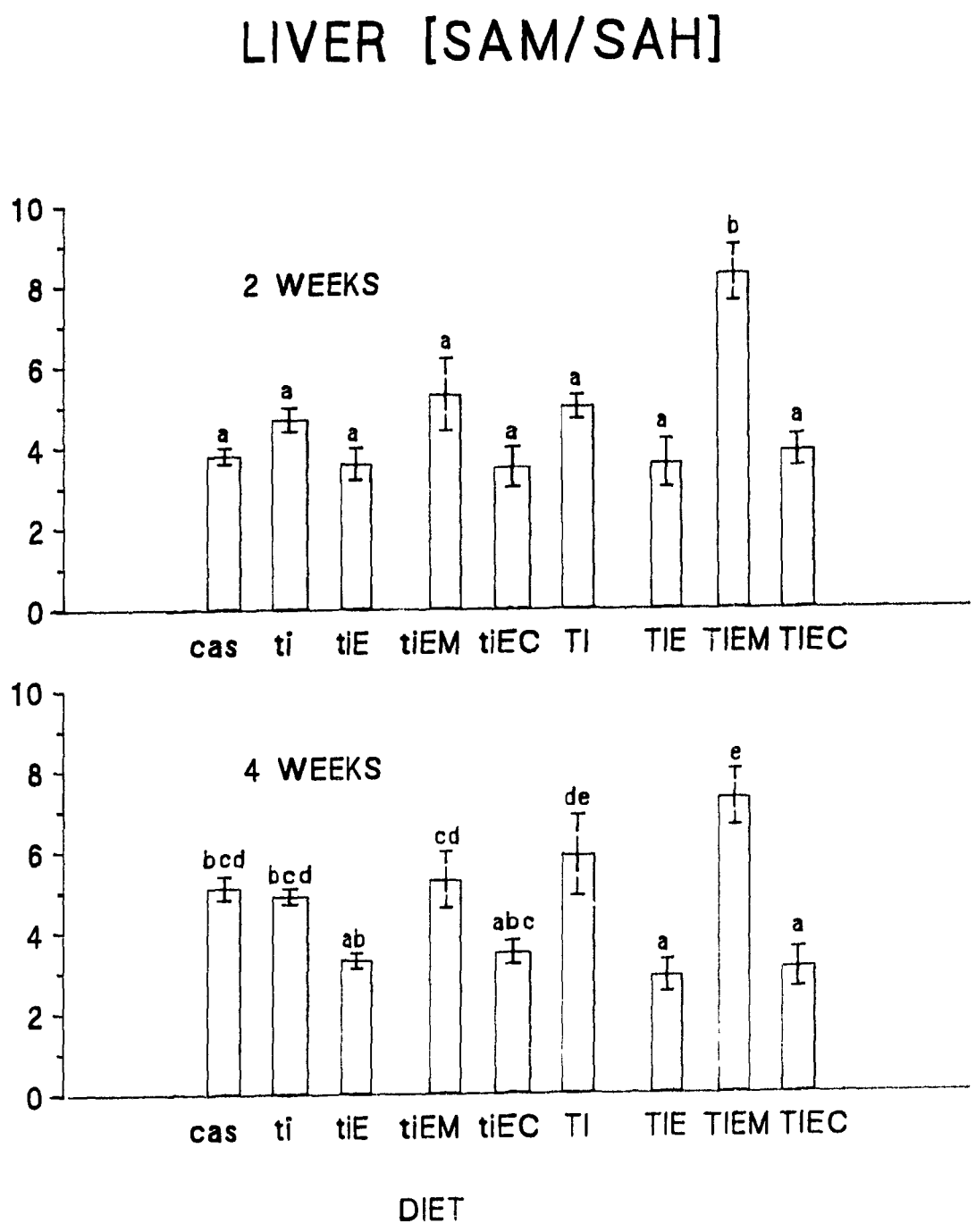

LIVER [SAM/SAH]
2 WEEKS
4 WEEKS
10
8
6
4
2
0
a
a
a
a
a
a
a
b
a
bcd
bcd
ab
cd
abc
de
a
e
a
cas
ti
tiE
tiEM
tiEC
TI
TIE
TIEM
TIEC
DIET

Figure 5.7 Liver S-adenosylethionine/S-adenosylmethionine ratios in rats fed diets differing in trypsin inhibitor content and presence or absence of supplementary sulfur amino acids over 2 and 4 weeks.

Bars representing treatment means with different letters at the same week, differ significantly ($P<0.05$).

Diet Key

cas: Casein control (0 trypsin inhibitor) (Diet 1)

ti : Low SBTI diet
(421 mg trypsin inhibited/100 g diet) (Diet 2)

tiE: Low SBTI diet + 0.2% DL-ethionine (Diet 3)

tiEM: Low SBTI diet + 0.2% DL-ethionine
+ L-methionine 3.35 mM/100 g diet) (Diet 4)

tiEC: Low SBTI diet + 0.2% DL-ethionine
+ L-cysteine (3.35 mM/100g diet) (Diet 5)

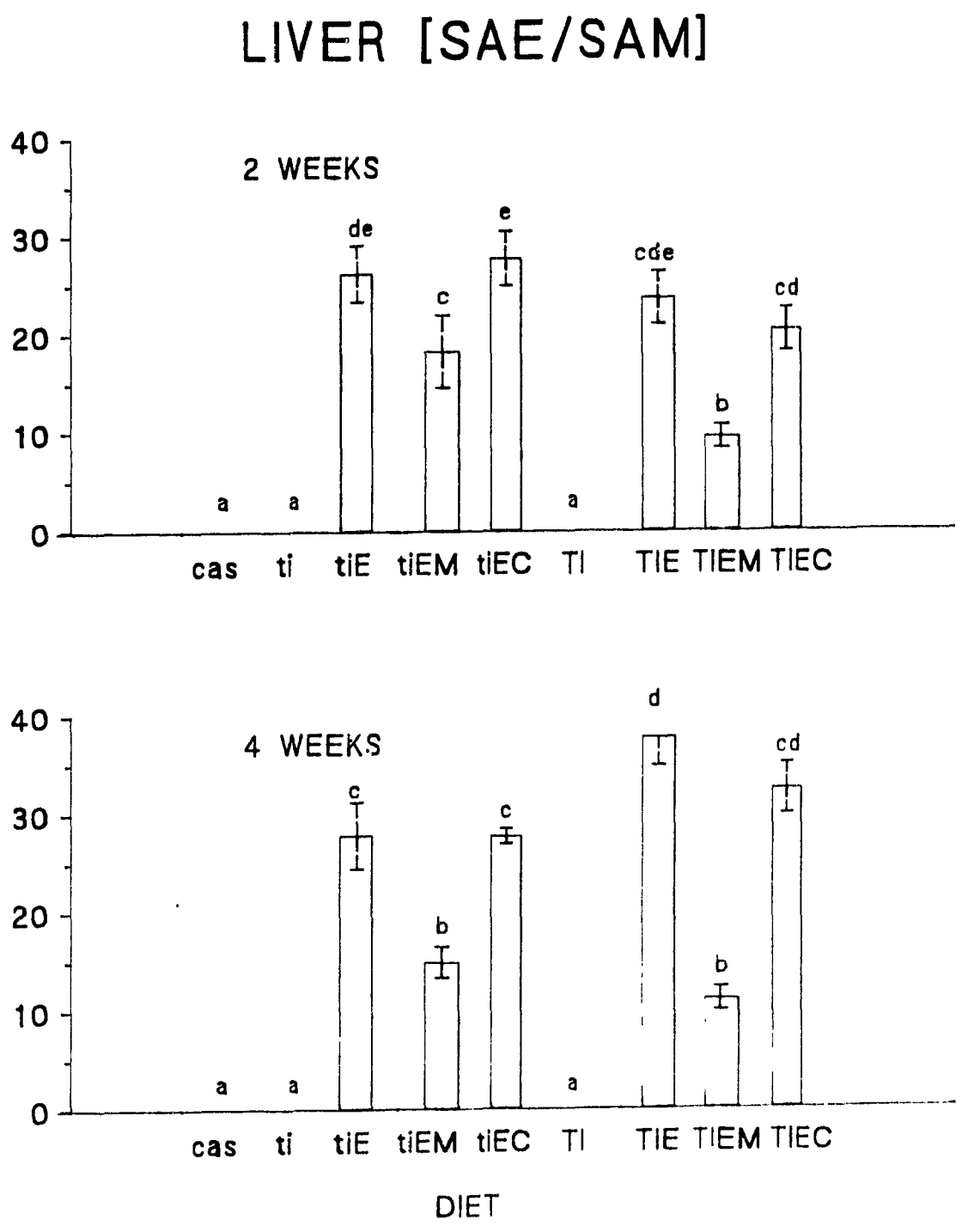
TI: High SBTI diet (775 mg trypsin inhibited/100g diet) (Diet 6)

TIE: High SBTI diet + 0.2% DL-ethionine (Diet 7)

TIEM: High SBTI diet + 0.2% DL-ethionine
+ L-methionine (3.35 mM/100g diet) (Diet 8)

TIEC: High SBTI diet + 0.2% DL-ethionine
+ L-cysteine (3.35 mM/100 g diet) (Diet 9)

Figure 5.7


LIVER [SAE/SAM]
2 WEEKS
40
30
20
10
0
a
a
de
c
e
a
cde
b
cd
cas
ti
tiE
tiEM
tiEC
TI
TIE
TIEM
TIEC
4 WEEKS
40
30
20
10
0
a
a
c
b
c
a
d
b
cd
cas
ti
tiE
tiEM
tiEC
TI
TIE
TIEM
TIEC
DIET

IV. GENERAL DISCUSSION

Soybeans have a tremendous potential towards meeting the protein needs of the world. They are the major source of high quality plant protein and edible oil in the United States (Smith and Circle 1972a) and their use is on the increase in North America (Wang 1984). Soy protein isolates can meet the protein maintenance requirements of the adult human and soy-based infant formulas are widely accepted and effective alternative sources of nutrition for human infants (Scrimshaw et al. 1983, Wink et al. 1985).

A variety of natural factors, present in food legumes can have antinutritional effects in animals (Gupta 1987). In the soybean, SBTI appear to be the predominant antinutrient. SBTI is a generic term for different classes of soy proteins that have the capacity to combine with and inactivate animal proteinases. Similar inhibitors exist in other food legumes and their capacity to restrict growth and cause pancreatic hypertrophy and hyperplasia in different animal species has been described (Rackis 1974, Rackis and Gumbmann 1981).

While SBTI are generally considered heat labile and almost completely destroyed by normal processing (Liener 1979, Rackis 1981) the general population consumes considerable quantities of proteinase inhibitors from both plant and animal sources (Doell et al. 1981) and residual quantities of active SBTI may exist in processed soy foods because of the need to balance SBTI destruction with maintenance of nutritional quality (Anderson et al. 1979). Certain risk groups may ingest even higher amounts of trypsin inhibitors and human infants fed soy-based formulas as a sole source of nutrition, may be particularly susceptible to the antinutrient effects of SBTI because of high intakes of soy products and because of relatively high amino acids requirement for growth.

No information is available on current residual levels of SBTI in soy-based infant formulas (Theuer and Sarett 1970, Churella et al. 1976) and there is an ongoing need to assess if improved methods of processing, newer formulations and a wider range of available soy-based products have influenced residual SBTI levels.

Using a modification of the standard enzymatic test for SBTI activity (Smith et al. 1980), it was found that currently available soy-based infant formulas retained residual levels of activity which were generally reflective of the low levels measured in soy protein isolates from which most soy-based formulas are derived. One product, however, retained up to 29% of the activity measured in a sample of raw soy that was also tested, suggesting that soy formula-fed infants could potentially consume considerable amounts of SBTI. Considerable variation existed between products and it would appear that the extent of processing was likely a factor in the observed differences. Ready-to-feed forms generally had highest levels of activity compared to concentrates and powdered forms from the same manufacturer. Greater heating of concentrates or powdered forms may have been involved. The predominance of more heat stable SBTI in some of the products may have occurred and there is a need to determine the different types of SBTI present because of the variable influence of different SBTI on human proteolytic activity. The monoclonal antibody techniques described by Brandon et al. (1988, 1989) may prove useful in this regard.

In addition to short-term antinutritional effects, SBTI have been shown to be involved in the long-term development of pancreatic cancer in laboratory animals with and without the presence of chemical carcinogens (McGuinness et al. 1984). SBTI have also been implicated in protection against certain cancers both in laboratory animals and in human epidemiological studies (Troll et al. 1980).

Rapidly dividing tissues are especially susceptible to the effects of various chemical carcinogens (Ryser 1971) and it may be that the hyperplastic effects of dietary SBTI may increase such susceptibility. While the mechanisms of such carcinogenesis are unknown, the relatively high exposure to SBTI of infants fed only soy-based products may be of special concern because of the already hyperplastic nature of tissue growth at this age. SBTI may render them even more susceptible to any environmental toxicants.

As already described, there are indications that inadequate intakes or increased requirements for dietary lipotropes can cause a variety of effects including depletion of labile methyl groups (Henning et al. 1979) and promotion or potentiation of cancers in animals (Newberne 1986). Alteration of nuclear DNA appears as the primary event in cancer development (Farber 1984) and it seems likely that the transmethylation/transsulfuration pathway, a fundamental process in which lipotropes are involved, may play a key role.

The first set of animal experiments in which rats were fed a low methionine diet based on raw ground soybeans containing SBTI, emphasized the importance of dietary methionine and pointed to certain clinical chemical parameters of methionine deficit and poor protein quality (elevated urea nitrogen and some indications of elevations of SGPT and alkaline phosphatase) that occurred with feeding SBTI. Serum lipids (total cholesterol and triglycerides) did not appear to change despite reports of a hypocholesterolemic effect of soy protein (Carroll 1983). Serum free amino acid changes (increase in serine, and decrease in taurine and low α-aminobutyric acid) as well as depressed growth, suggested inadequacy of dietary methionine supply. It had previously been demonstrated that poor growth, accumulation of amino acid substrates and decreased end products of the transsulfuration pathway, occurred when dietary methionine was insufficient (Peace et al. 1986). Addition of the high dose level of ethionine accentuated these various changes and indicated that the

use of ethionine (at somewhat lower dietary levels) combined with SBTI, would be an effective means of changing methionine status in young rats

The next experiment sought to look at potential changes occurring over a longer term in young male rats fed SBTI with and without ethionine when the dietary supply of methionine and other lipotropes was theoretically adequate for rat growth. Over a period of three months, increasing levels of dietary SBTI had a negative influence on growth and feed efficiency that was made worse by the presence of low levels of dietary ethionine. Serum free amino acid changes (accumulation of some essential amino acids and of non-essential amino acid substrates of the transsulfuration pathway) suggested that an ongoing deficit or decrease in the availability or utilization of methionine had occurred.

These findings have direct implications for infants consuming soy-based infant formulas. If product loyalty or price benefits dictate the exclusive use of a particular brand, there is a potential for continual exposure of infants to high SBTI intakes. With the known influence of SBTI on carcinogenesis in experimental animals and indications that humans and susceptible animals share common responses to SBTI, a diversity of foods for infants seems warranted.

Measurement of hepatic levels of SAM, SAH and SAE, and determination of ratios of SAM/SAH and SAE/SAM gave direct indications of the occurrence of methyl group deficit (Hoffman et al. 1978) in rats fed diets containing SBTI with and without ethionine. While similar compromise of methyl group availability has been demonstrated in studies of rats fed methionine or choline deficient diets (Finkelstein et al 1982c, Zeisel et al. 1989) this appears to be the first indication that SBTI present in soybeans can also induce such a deficit.

The interesting interactions between SBTI and ethionine in terms of clinical chemical and biochemical measures of lipotrope status and methyl donor adequacy reflect their influence on the transmethylation-transsulfuration pathway.

The relative effectiveness of methionine and cysteine in protecting against the negative growth, clinical chemical and biochemical changes occurring with SBTI and ethionine ingestion reflects the relative importance of the two amino acids in meeting sulfur amino acid requirements and their relative influence on the transmethylation-transsulfuration pathway.

V. GENERAL CONCLUSIONS

From the results obtained in these investigations the following conclusions can be made.

Despite newer formulations and newer methods of processing, residual levels of trypsin inhibitors continue to occur in soy-based infant formulas and can be present at relatively high levels compared to raw soybeans. The levels of SBTI in soy-based formulas vary in preparations from different manufacturers and in different preparation forms from the same manufacturer. This likely reflects processing differences and differences in types and levels of SBTI present in the soybeans selected for use.

Growth deficits and changes in clinical chemistry parameters in weanling or young rats soy-based diets high in trypsin inhibitors, indicate that SBTI induce signs of protein inadequacy (poor growth, decreased feed efficiency, reduced true protein digestibility) which can be made worse by the presence of carcinogenic levels of DL-ethionine, the ethyl analogue of methionine.

Changes in concentrations of serum free amino essential amino acids and non-essential amino acids related to the transsulfuration pathway of methionine in response to dietary SBTI or ethionine or combinations of the two, further indicate a deficit in protein synthesis and suggest the involvement of the transsulfuration pathway in the antinutrient and carcinogenic influences of SBTI.

Younger animals are especially sensitive to the influence of dietary SBTI and ethionine since many of the clinical indications of liver damage and abnormalities in lipid metabolism occurring with ingestion of these factors, moderated as the experiments progressed.

When weanling male rats were fed diets that were adequate for growth, the presence of dietary SBTI was still able to induce a sulfur amino acid deficit. The prolonged changes in clinical chemistry parameters and serum free amino acids, and the continuing growth deficits indicated that SBTI can have an ongoing influence in the growing rat. The presence of dietary SBTI can worsen the symptoms of lipotrope deficit induced by dietary ethionine, with the exception of liver fat deposition.

The failure to induce liver lipid deposition may be due to the hypolipidemic properties of soy protein.

The transmethylation-transsulfuration pathway of methionine metabolism is directly influenced by dietary SBTI and a methyl donor deficit appears to be a major feature of the symptoms induced by SBTI. Decreased hepatic concentrations of S-adenosylmethionine (SAM) and decreased ratios of S-adenosylmethionine to S-adenosylhomocysteine (SAM/SAH) clearly attest to the methyl donor deficit induced by dietary SBTI.

Increased ratios of S-adenosylethionine/SAM provide another indicator of deficit in the availability of SAM in the case of rats fed ethionine.

Methionine is a more effective supplement than cysteine in preventing or ameliorating many of the negative or abnormal growth, clinical chemistry and amino acid changes occurring with the methyl donor deficit induced by combination of SBTI and ethionine in the diet. This reflects their respective levels of input into the transmethylation-transsulfuration pathway.

BIBLIOGRAPHY

Abbey, B.W., Neale, R.J. and Norton, G. (1979a) Nutritional effects of field bean (Vicia faba L.) proteinase inhibitors fed to rats. Br. J. Nutr. 41: 31-38.

Abbey, B.W., Norton, R.J. and Neale, R.J. (1979b) Effects of dietary proteinase inhibitors from field bean (Vicia faba L.) and field-bean meal on pancreatic function in the rat. Br. J. Nutr. 41:39-45.

Adelson, J.W. and Rothman, S.S. (1974) Candidate hormones of the gut. J. Clin. Invest. 52: 1586-1589.

Alix, J.-H. (1982) Molecular aspects of the in vivo and in vitro effects of ethionine, an analog of methionine. Mirobiol. Reviews 46: 281-295.

Almquist H.J. and Merritt, J.B. (1953) Accentuation of dietary amino acid deficiency by raw soybean growth inhibitor. Proc. Soc. Exp. Biol. Med. 84:333-334.

American Institute of Nutrition (1977) Report of the American Institute of Nutrition Ad Hoc Committee on Standards for Nutritional Studies. J. Nutr. 117: 1340-1348.

Anderson, J.W. and Ward, K. (1979) High-carbohydrate, high fibre diets for insulin-treated men with diabetes mellitus. Amer. J. Clin. Nutr. 32: 2312-2321.

Anderson, R.L., Rackis, J.J. and Tallent, W.H. (1979). Biologically active substances in soy products. In: Soy Protein and Human Nutrition Wilke, H.L., Hopkins, D.T., and Waggle, D.H. (Eds.) pp. 209-233, Academic Press, New York.

Armstrong, B. and Doll, R. (1975) Environmental factors and cancer incidence and mortality in different countries with special reference to dietary factors. Int. J. Cancer 15: 617-631.

Ashton, F.M. (1976) Mobilization of storage proteins of seeds. Annu. Rev. Plant Physiol. 27. 95-117.

Atkinson, S.A. and Hanning, R.M. (1989) Amino acid metabolism and requirements of the premature infant: is human milk the "gold standard"? Chapter 14 in: Protein and Non-Protein Nitrogen in Human Milk, Atkinson, S.A. and Lonnerdal, B. (Eds.) pp. 187-209, CRC Press, Boca Raton, Florida.

Ausman, L.M., Harwood, J.P., King, N.W., Sehgal, P.K., Nicolosi, R.J., Hegsted, D.M., Liener, I.E., Donatucci, D. and Tarcza, J. (1985) The effects of long-term soy protein and milk protein feeding on the pancreas of Cebus albifrons monkeys. J. Nutr. 115: 1691-1701.

Ausman, L.M., Gallina, D.L., Hayes, K.C. and Hegsted, D.M. (1986) Comparative assessment of soy and milk protein quality in infant Cebus monkeys. Amer. J Clin. Nutr. 43: 112-127.

Bajjalieh, N., Orf, J.H., Hymowitz, T. and Jensen, A.H. (1980) Response of young chicks to raw, defatted, Kunitz trypsin inhibitor variant soybeans as sources of dietary protein. Poultry Sci. 59: 328-332.

Barnes, R.H. and Kwong, E. (1965) Effect of soybean trypsin inhibitor and penicillin on cystine biosynthesis in the pancreas and its transport as exocrine protein secretion in the intestinal tract of the rat. J. Nutr. 86: 245-252.

Barnes, R.H., Kwong, E. and Fiala, G. (1965) Effect of penicillin added to an unheated soybean diet on cystine excretion in feces of the rat. J. Nutr. 85: 123-126.

Becker, F.F. (1981) Inhibition of spontaneous hepatocarcinogenesis in C3H/HEN mice by Edipro A, an isolated soy protein. Carcinogenesis (Lond.) 2: 1213-1214.

Bender, A.E. (1987) Effects on nutritional balance: antinutrients. Chapter 4 in: Natural Toxicants in Food Progress and Prospects Watson-Weinheim, D.H. (Ed.) pp 110-124. New York: VCH; Chichester: Horwood; Horwood International Publications in Science and Technology. Chichester England.

Bhagavan, N.V. (1978) Biochemistry, 2nd Ed., pp. 424-455, J.P. Lippincott Co., Philadelphia, Toronto.

Bhat, V. A. and Pattabiraman, T.N. (1986) Natural plant enzyme inhibitors, action of seed proteinase inhibitors on ten different pancreatic preparations. J. Food Sci. Technol. (India) 23: 105-109.

Bhatty, R.S. and Christison, G.I. (1984) Composition and nutritional quality of pea (Pisum sativum L.), faba bean (Vicia faba L. spp. minor) and lentil (Lens culinaris Medik.) meals, protein concentrates and isolates. Qual. Plant. Plant Foods Hum. Nutr. 34: 41-51.

Bielorai, R., Harduf, Z. and Alumot, E. (1972) The free amino acid pattern of the intestinal contents of chicks fed raw and heated soybean meal. J. Nutr. 102: 1377-1382.

Bielorai, R., Tamir, M., Alumot, E., Bar, A. and Hurwitz, S. (1973) Digestion and absorption of protein along the intestinal tract of chicks fed raw and heated soybean meal. J. Nutr. 103: 1291-1298.

Birk, Y. (1965) Purification and properties of a highly active inhibitor of α-chymotrypsin from soybeans. Biochim. Biophys. Acta 54: 378-385.

Birk, Y. (1985) The Bowman-Birk inhibitor. Int. J. Peptide Protein Res. 25: 113-131.

Birk, Y., Gertler, A. and Khalef, S. (1963) A pure trypsin inhibitor from soybeans. Biochem J. 87: 281-284.

Birt, D.F., Salmasi, S., Pour, P.M. (1981) Enhancement of experimental pancreatic cancer in Syrian golden hamsters by dietary fat. J.Natl. Cancer Inst. 67: 1327-1332.

Bligh, E.G. and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37: 911-917.

Bodwell, C.E. (1975) Biochemical parameters as indices of protein nutritional value. Chapter 17 in: Protein Nutritional Quality of Foods & Feeds Vol. 1, Friedman, M. (Ed.), pp 261-310, Marcel Dekker Inc., New York.

Bodwell, C.E. (1978) Application of animal data to human protein nutrition: a review. Cereal Chem. 54: 958-983.

Booth, A.N., Robbins, D.J., Ribelin, W.E. and DeEds, F. (1960) Effect of raw soybean meal and amino acids on pancreatic hypertrophy in rats. Proc. Soc. Exp. Biol. Med. 104: 681-683.

Borchers, R. (1961) Counteraction of the growth depression of raw soybean oil meal by amino acid supplements in weanling rats. J. Nutr. 75: 330-334.

Borchers, R., Ackerson, C.W. and Mussehl, F.E. (1948) Trypsin inhibitor. VIII. Growth inhibiting properties of a soybean trypsin inhibitor. Arch. Biochem. Biophys. 19: 311-317.

Bornstein S. and Lipstein B. (1963) The influence of age of chicks on their sensitivity to raw soybean oil meal. Poultry Sci. 42: 61-70.

Bowman, E.E. (1946) Differentiation of soy bean antitryptic factors. Proc. Soc. Exp. Biol. Med. 63: 547-550.

Boyd, J.N., Graham, E.S., Graham, T.C., Tennant, B.C. (1985) A comparison of the response of woodchucks and rats to variations in dietary lipotrope and lipid content. J.Nutr. 5: 1136-1146.

Brada, Z., Bulba, S. and Altman, N.H. (1976) The influence of DL-methionine on the metabolism of S-adenosylethionine in rats chronically treated with DL-ethionine. Cancer Res. 36: 1573-1579.

Brandon, D.L., Bates, A.H. and Friedman, M. (1988) Enzyme-linked immunoassay of soybean Kunitz trypsin inhibitor using monoclonal antibodies. J. Food Sci. 53: 102-106.

Brandon, D.L., Bates, A.H., Friedman, M. (1989) Monoclonal antibody-based enzyme immunoassay of the Bowman-Birk protease inhibitor of soybeans. J. Agric. Food Chem. 37: 1192-1196.

Bressani, R. and Elias, L.G. (1974) New Protein Foods Vol. 1a, Altschul, A.M. (Ed.) p230, Academic Press, New York.

Broadway, R.M. and Duffey, S.S. (1986) Plant proteinase inhibitors: mechanism of action and effect on the growth and digestive physiology of larval Heliothis zea and Spodoptera exigua. J. Insect. Physiol. 32: 827-833.

Byington, M.H., Howe, J.M. and Clark, H.E. (1972) Effect of different levels and proportions of methionine, cystine, choline, and inorganic sulfur on growth and body composition of young rats. J. Nutr. 102: 219-228.

Calam, J., Bojarski, J.C. and Springer, C.J. (1987) Raw soya-bean flour increases cholecystokinin release in man. Br. J. Nutr. 58: 175-179.

Carroll, K.K. (1983) Dietary proteins and amino acids-their effects on cholesterol metabolism. Chapter 2 in Animal and Vegetable Proteins in Lipid Metabolism and Atherosclerosis, pp 9-17, Alan R. Liss Inc., New York.

Carroll, R.W., Hensley, G.W. and Graham, W.R. (1952) The site of nitrogen absorption in rats fed raw and heat-treated soybean meals. Science 115: 36-39.

Chan, J. and deLumen, B.O. (1982a) Properties of trypsin inhibitor from winged bean seed isolated by affinity chromatography. J. Agric. Food Chem. 30:42-46.

Chan, J. and deLumen, B.O. (1982b) Biological effects of isolated trypsin inhibitor from winged bean (Psophocarpus tetragonolubus) on rats. J. Agric Food Chem 30: 46-50.

Chen, I. and Mitchell, H.L. (1973). Trypsin inhibitors in plants. Phytochem. 12: 327-330.

Chernick, S.S., Lepkovsky, S. and Chaikoff, I.L. (1948) A dietary factor regulating the enzyme content of the pancreas: changes induced in size and proteolytic activity of the chick pancreas by the ingestion of raw soybean meal. Am. J. Physiol. 155: 33-41.

Chitra, R. and Sadasivam, S. (1986) A study of the trypsin inhibitor of Black Gram (Vigna mungo (L.) Hepper). Food Chem. 21: 315-320.

Churella, H.R., Yao, B.C. and Thomson, A.B. (1976) Soybean trypsin inhibitor activity of soy infant formulas and its nutritional significance for the rat. J. Agric. Food Chem. 24: 393-397.

Collins, J.L. and Sanders, G.G. (1976) Changes in trypsin inhibitor activity in some soybean varieties during maturation and germination. J. Food Sci. 41: 168-172.

Cooper, A.J.L. (1983) Biochemistry of sulfur-containing amino acids. Ann. Rev. Biochem. 52: 187-222.

Corring, T. (1974) Régulation de la sécrétion pancréatique par rétroaction négative chez le porc. Ann. Biol. Anim. Biochim. Biophys. 14: 487-498.

Corring, T. (1980) The adaptation of digestive enzymes to the diet: Its physiological significance. Reprod. Nutr. Devel. 20: 1217-1235.

Correa, P. (1981) Epidemiological correlations between diet and cancer frequency. Cancer Res. 41: 3685-3690.

Crass, R.A. and Morgan, R.G.H. (1981) Rapid changes in pancreatic DNA, RNA and protein in the rat during pancreatic enlargement and involution. Int J. Vitam. Res. 51: 85-91.

Crass, R.A. and Morgan, R.G.H. (1982) The effect of long-term feeding of soya-bean flour on pancreatic growth in the rat. Br. J. Nutr. 47: 119-129.

della Gatta, C., Piergiovanni, A.R. and Perrino, P. (1988) An improved method for determination of trypsin inhibitor levels in legumes. Lebensm.-Wiss. u.-Technol. 21: 315-318.

DiPietro, C.M. and Liener, I.E. (1989) Soybean protease inhibitors in foods. J. Food Sci. 54: 606-609, 617.

Doell, B.H., Ebden, C.J. and Smith, C.A. (1981) Trypsin inhibitor activity of conventional foods which are part of the British diet and some soya products. Qual. Plant Foods Hum. Nutr. 31: 139-150.

Duncan, D.B. (1955) Multiple range and multiple F tests. Biometrics 11: 1-42.

Eggum, B.O. (1970) Blood urea measurement as a technique for assessing protein quality. Brit. J. Nutr. 24: 983-988.

El-Hag, N. Haard, N.F. and Morse, R. E. (1978) Influence of sprouting on the digestibility coefficient, trypsin inhibitor and globulin proteins of red kidney beans. J. Food Sci. 43: 1874-1875.

Eloranta, T.O., Kajander, E.O. and Raina, A.M. (1976) A new method for the assay of tissue S-adenosylhomocysteine and S-adeonosylmethionine. Effect of pyridoxine deficiency on the metabolism of S-adenosylhomocysteine, S-adenosylmethionine and polyamines in rat liver. Biochem J. 160: 287-294.

Erdman, J.W. and Fordyce, E.J. (1989) Soy products and the human diet. Am. J. Clin. Nutr. 49: 725-737.

Eriksson, S. (1965) Studies in α_1-antitrypsin deficiency. Acta Med. Scand. 177 (Supp 432): 1-8.

FAO/WHO (1990) Report of the Joint FAO/WHO Expert Consultation on Protein Quality Evaluation. Food and Agricultural Organization of the United Nations. Rome, Italy.

FAO/WHO/UNU Expert Consultation (1985) Energy and Protein Requirements. WHO Technical Report Series No. 724. World Health Organization, Geneva, Switzerland.

Farber, E. (1963) Ethionine carcinogenesis. Adv. Cancer Res. 7: 383-474.

Farber, E. (1967) Ethionine fatty liver. Adv. Lipid Res. 5: 119-183.

Farber, E. (1984) The multistep nature of cancer development. Cancer Res. 44: 4217-4223.

Farber, E. and Ichinose, H. (1956) The prevention of ethionine-induced carcinoma of the liver in rats by ethionine. Cancer Res. 18: 1209-1213.

Feeney, R.E., Means, G.E. and Bigler, J.C. (1969) Inhibition of human trypsin, plasmin, and thrombin by naturally occurring inhibitors of proteolytic enzymes. J. Biol. Chem. 244: 1957-1960.

Feo, F., Garcea, R., Daino, L., Pascale, R., Pirisi, L., Frassetto, S. and Ruggiu, M.E. (1985) Early stimulation of polyamine biosynthesis during promotion by phenobarbital of diethylnitrosamine-induced rat liver carcinogenesis. The effects of variations of the S-adenosyl-L-methionine cellular pool. Carcinogenesis 6: 1713-1720.

Figarella C., Negri, G.A. and Guy, O. (1975) The two human trypsinogens. Inhibition spectra of the two human trypsins derived from their purified zymogens. Eur. J. Biochem 53: 457-463.

Finkelstein, J.D. (1990) Methionine metabolism in mammals. J. Nutr. Biochem. 1: 228-237.

Finkelstein, J.D. and Mudd, S.H. (1967) Trans-sulfuration in mammals. The methionine sparing effect of cystine. J. Biol. Chem. 242: 873-888.

Finkelstein, J.D., Harris, B.J., Martin, J.J. and Kyle, W.E. (1982a) Regulation of hepatic betaine-homocysteine methyltransferase by dietary methionine. Biochem. Biophys. Res. Commun. 108: 344-348.

Finkelstein, J.D., Kyle, W.E., Harris, B.J. and Martin, J.J. (1982b) Methionine metabolism in mammals: concentration of metabolites in rat tissues. J. Nutr. 112: 1011-1018.

Finkelstein, J.D., Martin, J.J., Harris, B. and Kyle, W.E. (1982c) Regulation of the betaine content of rat liver. Arch. Biochem. Biophys. 218: 169-173.

Finkelstein, J.D., Martin, J.J. and Harris, B. (1986) Effect of dietary cystine on methionine metabolism in rat liver. J. Nutr. 116: 985-990.

Finkelstein, J.D., Martin, J.J. and Harris, B. (1988) Effect of nicotinamide on methionine metabolism in rat liver. J. Nutr. 118: 828-833.

Fitzgerald, P.J. and Alvizouri, M. (1952) Rapid restitution of the rat pancreas following acinar cell necrosis subsequent to ethionine. Nature 170: 929-930.

Flavin, D.F. (1982) The effects of soybean trypsin inhibitors on the pancreas of animals and man: A review. Vet. Human Toxicol. 24: 25-28.

Folsch, K. Winkler, U.R. and Wormsley, K.G. (1974) Effect of soybean diet on enzyme content and ultrastructure of the rat exocrine pancreas. Digestion 11: 161-171.

Fomon, S.J., Ziegler, E.E., Nelson, S.E. and Edwards, B.B. (1986) Requirement for sulfur-containing amino acids in infancy. J. Nutr. 116: 1405-1422.

Freed, R.C. and Ryan, D.S. (1978) Changes in Kunitz trypsin inhibitor during germination of soybeans: an immunoelectrophoresis assay system. J. Food Sci. 43: 1316-1319.

Freed, R.C. and Ryan, D.S. (1980) Isolatation and characterizaton of genetic variants of the Kunitz soybean trypsin inhibitor. Biochim. Biophys. Acta 624: 562-572.

Friedman, M., Gumbmann, M.R. and Grosjean, O.K. (1984) Nutritional improvement of soy flour. J. Nutr. 114: 2241-2246.

Friedman, M. Gumbmann, M.R., Brandon, D.L. and Bates, A.H. (1989) Inactivation and analysis of soybean inhibitors of digestive enzymes. In: Food Proteins Kinsella, J.E. and Soucie, W.G. (Eds.) pp 296-328. American Oil Chemists Society, Champagne, IL.

Frost, A.B. and Mann, G.V. (1966) Effects of cystine deficiency and trypsin inhibitor on the metabolism of methionine. J. Nutr. 89: 49-54.

Fushiki, T. and Iwai, K. (1989) Two hypotheses on the feedback regulation of pancreatic enzyme secretion. Fed. Amer. Soc. Exp. Biol. J. 3: 121-126.

Gallaher, D. and Schneeman, B.O. (1984) Nutritional and metabolic response to plant inhibitors of digestive enzymes. In: Nutritional and Toxicological Aspects of Food Safety Freidman, M. (Ed.) pp. 299-320, Plenum Press, NY.

Gandhi, A.P., Nenwani, M.M. and Ali, N. (1984) Investigations on the trypsin inhibitor, urease and cooking behavior of soybean Glycine max Merr. Food Chem. 15: 215-218.

Gatehouse, J.A., Dobie, P., Kilminster, A.M. and Boulter, D. (1979) Biochemical basis of insect resistance in Vigna unguiculata. J. Sci. Food Agric. 30: 948-958.

Gennis, L.S. and Cantor, C.R. (1976) Double-headed protease inhibitors from black-eyed peas. I. Purification of two new protease inhibitors and the endogenous protease by affinity chromatography. J. Biol. Chem. 251: 734-740.

Gertler, A., Birk, Y. and Bondi, A. (1967) A comparative study of the nutritional and physiological significance of pure trypsin inhibitors and of ethanol-extracted soyabean meals in chicks and rats. J. Nutr. 91: 358-370.

Ghoshal, A.K., Ahluwalia, M. and Farber, E. (1983) The rapid induction of liver cell death in rats fed a choline-deficient methionine-low diet. Am. J. Pathol. 113: 309-314.

Ghoshal, A.K. and Farber, E. (1984) The induction of liver cancer by dietary deficiency of choline and methionine without added carcinogens. Carcinogenesis 5: 1367-1370.

Go, V.L.W., Hoffman, A.F. and Summerskill, W.H.J. (1970) Pancreozymin bioassay in man based on pancreatic enzyme secretion: potency of specific amino acids and other digestive products. J. Clin. Invest. 49: 1558-1564.

Goodale, R.L., Liener, I.E., Desmukh, A., Frick, T, Satterberg, T., Ward, G. and Borner, J.W. (1985) Do trypsin inhibitors affect feedback control of human pancreatic secretion? Digestive Diseases and Sciences 30: 972 (Abstract).

Gordon, R.K., Miura, G.A., Alonso, T. and Chaing, P.K. (1987) S-adenosylmethionine and its sulfur metabolites. Methods Enzymol. 143: 191-195.

Grant, G., McKenzie, N.H., Watt, W.B., Stewart, J.C., Dorward, P.M. and Pusztai, A. (1986) Nutritional evaluation of soya beans (Glycine max): nitrogen balance and fractionation studies. J. Sci. Food Agric. 37: 1001-1010.

Green G.M. and Lyman R.L. (1972) Feedback regulation of pancreatic enzyme secretion as a mechanism for trypsin inhibitor-induced hypersecretion in rats. Proc. Soc. Exp. Biol. Med. 140: 6-12.

Green, G.M., Olds, B.A., Matthews, G. and Lyman, R.L. (1973) Protein as a regulator of pancreatic enzyme secretion in the rat. Proc. Soc. Exp. Biol. Med. 142: 1162-1167.

Green, G.M., Levan, V.H. and Liddle, R.A. (1986) Interaction of dietary protein and trypsin inhibitor on plasma cholecystokinin and pancreatic growth in rats. Adv. Exp. Med. Biol. 199: 123-132.

Green N.M. and Work, E. (1953) Pancreatic trypsin inhibitor 2. Reaction with trypsin. Biochem. J. 54: 347-352.

Green, T.R. and Ryan, C.A. (1972) Wound-induced proteinase inhibitor in plant leaves: a possible defense mechanism against insects. Science 175: 776-777.

Gumbmann, M.R. and Friedman, M. (1987) Effect of sulfur amino acid supplementation of raw soy flour on the growth and pancreatic weights of rats. J.Nutr. 117: 1018-1023.

Gumbmann, M.R., Spangler, W.L., Dugan, G.M., Rackis, J.J. and Liener, I.E. (1985) The USDA trypsin inhibitor study. IV. The chronic effects of soy flour and soy protein isolate on the pancreas in rats after two years. Qual. Plant Foods Hum. Nutr. 35: 275-314.

Gumbmann, M.R., Spangler, W.L., Dugan, G.M. and Rackis, J.J. (1986) Safety of trypsin inhibitors in the diet: effects of long-term feeding of soy flour and soy protein isolate. Adv. Exp. Med. Biol. 199: 33-79.

Gumbmann, M.R., Dugan, G.M., Spangler, W.L., Baker, E.C. and Rackis, J.J. (1989) Pancreatic response in rats and mice to trypsin inhibitors from soy and potato after short- and long-term dietary exposure. J. Nutr. 119: 1598-1609.

Gupta, Y.P. (1987) Anti-nutritional and toxic factors in food legumes: a review. Plant Foods Hum. Nutr. 37: 201-228.

Hamilton, R.M.G. and Carroll, K.K. (1976) Plasma cholesterol levels in rabbits fed low fat, low cholesterol diets. Effects of dietary proteins, carbohydrates, and fibre from different sources. Atherosclerosis 24: 47-62.

Hammerstrand, G.E., Black, L.T. and Glover, J.D. (1981) Trypsin inhibitors in soy products: modification of the standard analytical procedure. Cereal Chemistry 58: 42-45.

Harper, A.E., Benevenga, N.J. and Wohlhueter, R. (1970) Effects of ingestion of disproportionate amounts of amino acids. Physiol. Rev. 50: 428-558.

Hasdai, A. and Liener, I.E. (1983) Growth, digestibility and enzymatic activities in the pancreas and intestines of hamsters fed raw and heated soy flour. J.Nutr. 113: 662-668.

Hasdai, A., Nitsan, Z., Volcani, R. and Birk, Y. (1989) Growth, digestibility, and enzyme activities in the pancreas and intestines of guinea-pigs fed on raw and heated soya-bean flour. Br. J. Nutr. 62: 529-537.

Hartl, P., Tan-Wilson, A.L. and Wilson, K.A. (1986) Proteolysis of Kunitz soybean trypsin inhibitor during germination. Phytochem. 25:23-26.

Haynes, R. and Feeney, R.E. (1967) Fractionation and properties of trypsin and chymotrypsin inhibitors from lima beans. J. Biol. Chem. 242: 5378-5375.

Health Protection Branch (1988) Protocol for projects involving the use of laboratory animals. Animal Care Committee, Health Protection Branch, Health and Welfare Canada.

Henning, S.M., McKee, R.W. and Swendseid, M.E. (1989) Hepatic content of S-adenosylmethionine, S-adenosylhomocysteine and glutathione in rats receiving treatments modulating methyl donor availability. J. Nutr. 119: 1478-1482.

Hilder, V.A., Gatehouse, A.M., Sheerman, S.E., Barker, R.F. and Boulter, D. (1987) A novel mechanism of insect resistance engineered into tobacco. Nature 330: 160-163.

Hiriyama, T. (1979) Diet and cancer. Nutr. Cancer 1: 67-81.

Hoffman, R.M. (1984) Altered methionine metabolism, DNA methylation and oncogene expression in carcinogenesis. Bichim. Biophys. Acta 738: 49-87.

Hoffman, D.R., Cornatzer, W.E. and Duerre, J.A. (1978) Relationship between tissue levels of S-adenosylmethionine, S-adenosylhomocysteine, and transsulfuration reactions. Can. J. Biochem. 57: 56-65.

Howatson, A.G. (1986) Pancreatic carcinogenesis - the potential of cholecystokinin as a cocarcinogen in the hamster-nitrosamine model. Adv. Exp. Med. Biol. 199: 109-121.

Hwang, D.L.-R., Davis Lin, K.-T., Yang, W.-K. and Foard, D.E. (1977) Purification, partial characterization, and immunological relationships of multiple low molecular weight protease inhibitors of soybean. Biochim. Biophys. Acta 495: 369-382.

Hyde, C.L. and Poirier, L.A. (1982) Hepatic levels of S-adenosylethionine and S-adenosylmethionine in rats and hamsters during subchronic feeding of DL-ethionine. Carcinogenesis 3: 309-312.

Hymnowitz, W. (1986) Genetics and breeding of soybeans lacking the Kunitz trypsin inhibitor. Adv. Exp. Med. Biol. 199: 291-298.

Ihse, I., Arnesjo, B. and Lundquist, I. (1974) Glucose-induced insulin secretion and pattern of exocrine pancreatic enzymes in the rat following oral and parenteral trypsin inhibitor administration. Scand. J. Gastroent. 9: 719-724.

Ihse, I., Lilja, P. and Lundquist, I. (1977) Feedback regulation of pancreatic enzyme secretion by intestinal trypsin in man. Digestion 15: 303-308.

James, K.A.C. and Hove, E.L. (1980) The ineffectiveness of supplementary cystine in legume-based rat diets. J. Nutr. 110: 1736-1744.

Janzen, D.H., Juster, H.B. and Liener, I.E. (1976) Insecticidal action of the phytohemagglutinin in black beans on a bruchid beatle. Science 192: 795-796.

Janzen, D.H., Ryan, C.A., Liener, I.E. and Pearce, G. (1986) Potentially defensive proteins in mature seeds of 59 species of tropical leguminosae. J. Chem. Ecol. 12: 1469-1480.

Kadam, S.S. and Smithard, R.S. (1987) Effects of heat treatments on trypsin inhibitor and hemagglutinating activities in winged bean. Plant Foods Hum. Nutr. 37: 151-159.

Kakade, M.L., (1974) Biochemical basis for the differences in plant protein utilization. J. Agr. Food Chem. 22: 550-555.

Kakade, M.L., Barton, T.L., Schaible, P.J. and Evans, R.J. (1967) Biochemical changes in the pancreases of chicks fed raw soybeans and soybean meal. Poultry Sci. 46: 1578-1580.

Kakade, M.L., Arnold, R.L. , Liener, I.E. and Waibel, P.E. (1969) Unavailability of cystine from trypsin inhibitor as a factor contributing to the poor nutritive value of navy beans. J. Nutr. 99: 34-42.

Kakade, M.L., Simons, M.R., Liener, I.E. and Lambert, J.W. (1972) Biochemical and nutritional assessment of different varieties of soybeans. J. Agric. Food Chem. 20: 87-90.

Kakade, M.L., Hoffa, D.E. and Liener, I.E. (1973) Contribution of trypsin inhibitors to the deleterious effects of unheated soybeans fed to rats. J.Nutr. 103: 1772-1778.

Kakade, M.L., Rackis, J.J., McGhee, J.E. and Puski, G. (1974) Determination of trypsin inhibitor activity of soy products: a collaborative analysis of an improved procedure. Cereal Chem. 51: 376-382.

Kakade, M.L., Thompson, R.D., Engelstad, W.E., Behrens, G.C.. Yoder, R.D. and Crane, F.M. (1976) Failure of soybean trypsin inhibitor to exert deleterious effects in calves. J.Dairy Sci. 59: 1484-1489.

Kamalakannan, V., Sathyamoorthy, A.V. and Motlag, D.B. (1981) Studies on black gram (Vigna mungo) trypsin inhibitor. J. Sci. Food Agric. 32: 1172-1176.

Kanduc, D., Ghoshal, A., Quagliariello, E. and Farber, E. (1988) DNA hypomethylation in ethionine-induced rat preneoplastic hepatocyte nodules. Biochim. Biophys. Res. Comm. 150: 739-744.

Keith, M.O., Christensen, D.A., Owen, B.D. (1972) Determination of the methionine requirement of growing pigs using serum free amino acids. Can. J. Anim. Sci. 52: 153-169.

Kennedy, A.R. and Little, J.B. (1981) Effects of protease inhibitors on radiation transformation in vitro. Cancer Res. 41: 2103-2108.

Kim, S., Hara, S., Hase, S., Ikenaka, T., Toda, H., Kitamura, K. and Kaizuma, N. (1985) Comparative study on amino acid sequences of Kunitz-type soybean trypsin inhibitors, Ti^a, Ti^b, and Ti^c. J. Biochem. 98: 435-448.

Kortt, A. A. (1979) Isolation and characterization of the trypsin inhibitors from winged bean seed (Psophocarpus tetragonolobus (L.) Dc.). Biochim. Biophys. Acta 577: 371-382.

Krogdahl A. and Holm, H. (1979) Inhibition of human and rat pancreatic proteinases by crude and purified soybean proteinase inhibitors. J. Nutr. 109: 551-558.

Krogdahl, A. and Holm, H. (1981) Soybean proteinase inhibitors and human proteolytic enzymes: selective inactivation of inhibitors by treatment with human gastric juice. J. Nutr. 111: 2045-2051.

Krogdahl, A. and Holm, H. (1982) Activation and pattern of proteolytic enzymes in pancreatic tissue from rat, pig, cow, chicken, mink and fox. Comp. Biochem. Physiol. 72A: 575-578.

Krogdahl, A. and Holm, H. (1983) Pancreatic proteinases from man, trout, rat, pig, cow, chicken, mink and fox. Enzyme activities and inhibition by soybean and lima bean proteinase inhibitors. Comp. Biochem. Physiol. 74B: 403-409.

Kunitz, M. (1947) Crystalline soybean trypsin inhibitor II. General properties. J. Gen. Physiol. 30: 291-310.

Kwong, E. and Barnes, R.H. (1963) Effect of soybean trypsin inhibitor on methionine and cystine utilization. J. Nutr. 81: 392-398.

Kwong, E., Barnes, R.H. and Fiala, G. (1962) Intestinal absorption of nitrogen and methionine from processed soybeans in the rat. J. Nutr. 77: 312-316.

Laskowski, M., Jr. (1986) Protein inhibitors of serine proteinases-mechanism and classification Adv. Exp. Med. Biol. 199: 1-18.

Laskowski, M., Jr. and Kato, I. (1980) Protein inhibitors of proteinases. Annu. Rev. Biochem. 49: 593-626.

Levison, D.A., Morgan, R.G.H., Brimacombe, J.S., Hopwood, D., Coghill, G. and Wormsley, K.G. (1979) Carcinogenic effects of di(2-hydroxypropyl) nitrosamine (DHPN) in male Wistar rats: promotion of pancreatic cancer by raw soya flour diet. Scand. J. Gastroenterol. 14: 217-224.

Lewis, J.H. and Taylor, F.H.L. (1947) Comparative utilization of raw and autoclaved soy bean protein by the human. Proc. Soc. Exp. Biol. Med. 64: 85-87.

Liddle, R.A., Green, G M. Conrad, C.K. and Williams, J.A. (1986) Proteins but not amino acids, carbohydrates, or fats stimulate cholecystokinin secretion in the rat. Am. J. Physio. 251: G243-G248.

Liener, I.E. (1962) Toxic factors in edible legumes and their elimination. Am. J. Clin. Nutr. 11: 281-298.

Liener, I.E. (1974) Phytohemagglutinins: Their nutritional significance. J. Agr. Food Chem. 22: 17-22.

Liener, I.E. (1976) Legume toxins in relation to protein digestibility - a review. J. Food Sci. 41: 1076-1081.

Liener, I.E. (1977) Toxicological considerations in the utilization of new protein foods. In: Biochemical Aspects of New Protein Foods J. Adler-Nissen, (Ed.) pp. 129-138, FEBS, Copenhagen, Denmark.

Liener, I.E. (1979). Significance for humans of biologically active factors in soybeans and other food legumes. J. Am. Oil Chemists Soc. 56: 121-129.

Liener, I.E. (1990) Antinutritional factors. Chapter 10 in: Legumes. Chemistry, Technology and Human Nutrition, Matthews, R.H. (Ed.) pp339-382. Marcel Dekker Inc. New York and Basel.

Liener, I.E. and Fevold, H.L. (1949) The effect of soybean trypsin inhibitor on the enzymatic release of amino acids from autoclaved soybean meal. Arch. Biochem. Biophys. 21:395-307.

Liener, I.E. and Hasdai, A. (1986) The effect of long-term feeding of raw soyflour on the pancreas of the mouse and hamster. Adv. Exp. Med. Biol. 199: 189-197.

Liener, I.E. and Kakade, M.L. (1980) Protease inhibitors. In: Toxic Constituents of Plant Foodstuffs. Liener, I.E. (Ed.) pp7-72. Academic Press, New York.

Liener, I.R., Nitsan, Z., Srisangnam, C., Rackis, J.J. and Gumbmann, M.R. (1985) The USDA trypsin inhibitor study. II. Timed related biochemical changes in the pancreas of rats. Qual. Plant Foods Hum. Nutr. 35: 243-257.

Liener, I.R., Goodale, R.L. Deshmukh, A., Satterberg, T.L., Ward, G., DiPietro, C.M., Bankey, P.E., Borner, J.W. (1988) Effect of a trypsin inhibitor from soybeans (Bowman-Birk) on the secretory activity of the human pancreas. Gastroenterology 94: 419-427.

Lombardi, B. (1971) Effects of choline deficiency on rat hepatocytes. Fed. Proc. 30: 139-142.

Loo, G. and Smith, J.T. (1986) Regulation of rat liver protein methylase III by S-adenosylhomocysteine after D,L-homocysteine thiolactone administration and during pyridoxine deficiency. Nutr. Res. 6: 225-231.

Lorensen, E., Prevosto, R. and Wilson, K.A. (1981) The appearance of new active forms of trypsin inhibitor in germinating mung bean (Vigna radiata) seeds. Plant Physiol. 68: 88-92.

Mack, T.M. (1982) Cancer Epidemiology and Prevention, Schottenfeld, D. and Fraumeni, J. (Eds.) pp638-667, W.B.Saunders Co., Philadelphia, PA.

Mack, T.M., Yu, M.C., Hanisch, R. and Henderson, B.E. (1986) Pancreas cancer and smoking, beverage consumption, and past medical history. J. Nat. Cancer Inst. 1: 49-60.

Madden, M.A., Tan-Wilson, A.L. and Wilson, K.A. (1985) Proteolysis of soybean Bowman-Birk trypsin inhibitor during germination. Phytochem. 24: 2811-2815.

Mallory, P.A. and Travis, J. (1975) Inhibition spectra of the human pancreatic endopeptidases. Am. J. Clin. Nutr. 28: 823-830.

Marquardt, R.R., McKirdy, J.A., Ward, T. and Campbell, L.D. (1975) Amino acid, hemagglutinin and trypsin inhibitor levels, and proximate analyses of faba beans (Vicia faba) and faba bean fractions. Can. J. Anim. Sci. 55: 421-429.

McGuinness, E.E., Morgan, R.G.H., Levison, D.A., Frape, D.L., Hopwood, D. and Wormsley, K.G. (1980) The effects of long-term feeding of soya flour on the rat pancreas. Scand. J Gastroenterol. 15:497-502.

McGuinness, E.E., Morgan, R.G.H., Levison, D.A., Hopwood, D. Wormsley, K.G. (1981) Interaction of azaserine and raw soya flour on the rat pancreas. Scand. J. Gastroenterol. 16: 49-56.

McGuinness, E.E, Hopwood, D. and Wormsley, K.G. (1982) Further studies of the effects of raw soya flour on the rat pancreas. Scand. J. Gastroenterol. 17:273-277.

McGuinness, E., Morgan, R.G.H. and Wormsley, K.G. (1984) Effects of soybean flour on the pancreas of rats. Environ. Health Perspect. 56: 205-212.

McLaughlan, J.M. (1963) Relationship between protein quality and plasma amino acid levels. Fed. Proc. 22: 1122-29.

McNab, J.M. (1977) Response of broiler chickens to heat treatment of field beans (Vicia faba L.). In: Protein Quality From Leguminous Crops pp 236-242. Commission of European Communities, Luxembourg: Office for Official Publications of the European Communities.

McNaughton, J.L. and Reece, F.N. (1980) Effect of moisture content and cooking time on soybean meal urease index, trypsin inhibitor content, and broiler growth. Poultry Sci. 59: 2300-2306.

McNaughton, J.L., Reece, F.N. and Deaton, J.W. (1981) Relationships between color, trypsin inhibitor contents, and urease index of soybean meal and effects on broiler growth. Poultry Sci. 60: 393-400.

Meister, A. (1988) Glutathione metabolism and its selective modification. J. Biol. Chem. 268: 17205-17208.

Melmed, R.N., El-Asser, A.A.A. and Holt, S.J. (1976) Hypertrophy and hyperplasia of the neonatal rat exocrine pancreas induced by orally administered soybean trypsin inhibitor. Biochim. Biophys. Acta 421: 280-288.

Melnick, D., Oser, B.L. and Weiss, S. (1946) Rate of enzymic digestion of proteins as a factor in nutrition. Science 103: 326-329.

Meyer, J.H. and Jones, R.S. (1974) Canine pancreatic responses to intestinally perfused fat and products of fat digestion. Am. J. Physiol. 226: 1178-1187.

Meyer, J.H., Kelley, G.A., Spingola, L.J. and Jones, R.S. (1976) Canine gut receptors mediating pancreatic responses to luminal L-amino acids. Am. J. Physiol. 231: 669-677.

Mikol, Y.B., Hoover, K., Creasia, D. and Poirier, L.A. (1983) Hepatocarcinogenesis in rats fed methyl-deficient, amino acid-defined diets. Carcinogenesis 4: 1619-1629.

Mikola, J. and Kirsi, M. (1972) Differences between endospermal and embryonal trypsin inhibitors in barley, wheat, and rye. Acta Chem. Scand. 26: 787-795.

Minakata, K., Asano, M., Sato, T. and Harada, N. (1982) Assay of a-cysteine proteinase inhibitor in serum or plasma. Hoppe-Seyler's Z. Physiol. Chem. 363: 493-498.

Minakata, K., Asano, M., Takahashi, Y. and Harada, N. (1987) Enhancement of testicular cysteine proteinase inhibitor level in Vitamin E-deficient rats. J. Nutr. 1416-1421.

Miyasaka, K. and Green, G.M. (1983) Effect of rapid washout of proximal small intestine on pancreatic secretion in conscious rat. Gastroenterol. 84: 1251 (Abstract)

Morgan, R.G.H. and Wormsley, K.G. (1977) Cancer of the pancreas. Gut 18: 580-596.

Morgan, R.G.H., Levison, D.A., Hopwood, D., Saunders, J.H. and Wormsley, K.G. (1977) Potentiation of the action of azaserine on the rat pancreas by raw soya bean flour. Cancer Letters 3: 87-90.

Morgan, R.G.H., Crass, R.A. and Oates, P.S. (1986) Dose effects of raw soybean flour on pancreatic growth. Adv. Exp. Med. Biol. 206: 81-89.

Nagata, Y., Imaizumi, K. and Sugano, M. (1980) Effect of soyabean protein and casein on serum cholesterol levels in rats. Br. J. Nutr. 44: 113-121.

National Research Council (1978) Nutrient Requirements of Laboratory Animals, Third Revised Edition. National Academy of Sciences, Washington DC.

Newberne, P.M. (1986) Lipotropic factors in oncogenesis. Adv. Exp. Med. Biol. 206: 223-251.

Newberne, P.M. and Conner, M.W. (1986) Nutrient influences on toxicity and carcinogenicity. Fed. Proc. 45: 149-154.

Newberne, P.M., Rogers, A.E. and Wogan, G.N. (1968) Hepatorenal lesions in rats fed a low lipotrope diet and exposed to aflatoxin. J. Nutr. 94: 332-343.

Newberne, P.M., Rogers, A.E. and Nauss, K.M. (1983) Choline, methionine, and related factors in oncogenesis. Chapter 15 in: Nutrition Factors in the Induction and Maintenance of Malignancy, pp. 247-271, Academic Press Inc., NY.

Nielsen, S.S. and Liener, I.E. (1984) Degradation of the major storage protein of Phaseolus vulgaris during germination. Plant Physiol. 74: 494-498.

Nielsen, S.S. and Liener, I.E. (1988) Effect of germination on trypsin inhibitor and hemagglutinating activities in Phaseolus vulgaris. J. Food Sci. 53: 298-301.

Niess, E., Ivy, C.A. and Nesheim, M.C. (1972) Stimulation of gallbladder emptying and pancreatic secretion in chicks by soybean whey proteins. Proc. Soc. Exp. Biol. Med. 140: 291-296.

Nitsan, Z. and Alumot, E. (1964) Overcoming the inhibition of intestinal proteolytic activity caused by raw soybean in chicks of different ages. J. Nutr. 87: 179-184.

Nitsan, Z. and Liener, I.E. (1976a) Studies of the digestibility and retention of nitrogen and amino acids in rats fed raw or heated soy flour. J. Nutr. 105: 292-299.

Nitsan, Z. and Liener, I.E. (1976b) Enzymic activities in the pancreas, digestive tract and feces of rats fed raw or heated soy flour. J. Nutr. 106: 300-305.

Nitsan, Z. and Nir, I. (1977) A comparative study of the nutritional and physiological significance of raw and heated soya-beans in chicks and goslings. Br. J. Nutr. 37: 86-97.

Nitsan, Z. and Nir. I. (1986) Accentuated response to dietary raw soy flour by meal feeding in various species. Adv. Exp. Med. Biol. 199: 199-222.

Nitsan, Z., Nir, I. and Liener, I.E. (1983) Accentuated response to raw soya-bean meal by meal feeding. Arch. Toxicol., Suppl. 6: 177-181.

Oates, P.S. and Morgan, R.G.H. (1984) Short-term effects of feeding raw soya flour on pancreatic cell turnover in the rat. Am. J. Physiol. 247: G667-G673.

Obara, T. and Watanabe Y. (1971) Heterogeneity of soybean trypsin inhibitors. 2. Heat inactivation. Cereal Chem. 48: 523-527.

Odani, S. and Ikenaka, T. (1976) The amino acid sequences of two soybean double headed proteinase inhibitors and evolutionary consideration of the legume proteinase inhibitors. J. Biochem. (Tokyo) 80: 641-643.

Odani, S., Koide, T. and Ono, T. (1983) A possible evolutionary relationship between plant trypsin-inhibitor, alpha amylase inhibitor and mammalian pancreatic secretory trypsin-inhibitor (Kazal). J. Biochem. 93: 1701-1704.

Oden, K.L. and Clarke, S. (1983) S-adenosyl-L-methionine synthetase from human erythrocytes: role in regulation of cellular S-adenosylmethionine levels. Biochem. 22: 2978-2986.

Ogun, P.O., Markakis, P. and Chenoweth, W. (1989) Effect of processing on certain antinutrients in cowpeas (Vigna unguiculata). J. Food Sci. 54: 1084-1085.

Osborne, T.B. and Mendel, L.B. (1917) The use of soy bean as food. J. Biol. Chem. 32: 369-387.

Palmer, R., McIntosh, A. and Pusztai, A. (1973) The nutritional evaluation of kidney beans (Phaseolus vulgaris). The effect on nutritional value of seed germination and changes in trypsin inhibitor content. J. Sci. Fd. Agric. 24: 937-944.

Pascale, R., Pirisi, L., Daino, L., Zanetti, S., Sata, A., Bartoli and Feo, F. (1982) Role of phosphatidylethanolamine methylation in the synthesis of phosphatidylcholine by hepatocytes isolated from choline-deficient rats. FEBS Lett. 145: 293-297.

Patten, J.R., Richards, E.A. and Pope, H. (1971) The effect of raw soybean on the pancreas of adult dogs. Proc. Soc. Exp. Biol. Med. 137: 59-63.

Peace, R.W., Sarwar, G., Botting, H.G. and Chavez, E.R. (1986) Sulfur amino acid requirements of the growing rat fed eight percent dietary protein. Nutr. Res. 6: 295-307.

Phillips, R.L. (1975) Role of life-style and dietary habits in risk of cancer among Seventh-Day Adventists. Cancer Res. 35: 3513-3522.

Poirier, L.A., Grantham, P.H. and Rogers, A.E. (1977) The effects of a marginally lipotrope-deficient diet on hepatic levels of S-adenosylmethionine and on the urinary metabolites of 2-acetylaminofluorene in rats. Cancer Res. 37: 744-748.

Poort, S.R. and Poort, C. (1981) Effect of feeding diets of different composition on the protein synthetic pattern of the rat pancreas. J. Nutr. 111: 1474-1479.

Prabhu, K.S., Saldanha, K. and Pattabiraman, N. (1984) Natural plant enzyme inhibitors: a comparative study of the action of legume inhibitors on human and bovine pancreatic proteinases. J. Sci. Food Agric. 35: 314-321.

Protein Advisory Group of the United Nations, (1973) PAG Statement #22, PAG Bull., Vol. III, No. 2, United Nations, New York.

Pusztai, A. (1972) Metabolism of trypsin-inhibitory proteins in the germinating seeds of kidney bean. Planta 107: 121-127.

Rackis, J.J. (1965) Physiological properties of soy bean trypsin inhibitors and their relationship to pancreatic hypertrophy and growth inhibition of rats. Fed. Proc. 24: 1488-1493.

Rackis, J.J. (1966) Soybean trypsin inhibitors: their inactivation during meal processing. J. Food Technol. 20: 1482-1488

Rackis, J.J. (1972) Biologically active components. In: Soybeans. Chemistry and Technology, Vol 1, Proteins Smith, A.K, and Circle, S.J. (Eds.) pp 158-202. Avi Publ. Co. Westport, Conn.

Rackis, J.J. (1974) Biological and physiological factors in soybeans. J. Am. Oil Chem. Soc. 51: 161A-174A.

Rackis, J.J. (1981) Significance of trypsin inhibitors in nutrition. Am. J. Oil Chem. Soc. 56: 495-501.

Rackis, J.J. and Gumbmann, M.R. (1981) Protease inhibitors: physiological properties and nutritional significance. Chapter 12 in: Antinutrients And Natural Toxicants In Foods Ory, R.L (Ed.) pp. 203-237, Food and Nutrition Press Inc., Westport, CT.

Rackis, J.J., Smith, A.K., Nash, A.M., Robbins, D.J. and Booth, A.N. (1963) Feeding studies on soybeans. Growth and pancreatic hypertrophy in rats fed soybean meal fractions. Cereal Chem. 40: 531-538.

Rackis, J.J., McGhee, J.E. and Booth, A.N. (1975) Biological threshold levels of soybean trypsin inhibitor by rat bioassay. Cereal Chem 52: 85-92.

Rackis, J.J., Gumbmann, M.R. and Liener, I.E. (1985) The USDA trypsin inhibitor study. I. Background, objectives and procedural details. Qual. Plant Foods Hum. Nutr. 35: 213-242.

Rackis, J.J., Wolf, W.J. and Baker, E.C. (1986) Protease inhibitors in plant foods: content and inactivation. Adv. Exp. Med. Biol. 199: 299-347.

Rao, B.S.N. and Prabhavathi, T. (1982) Tannin content of foods commonly consumed in India and its influence on ionisable iron. J. Sci. Food Agric. 33: 89-96.

Rao, P.B. Rama, Metta, V.C. and Johnson, B.C. (1959) The amino acid composition and the nutritive value of proteins. I. Essential amino acid requirements of the growing rat. J. Nutr. 69: 387-391.

Reddy, N.R., Pierson, M.D. and Sathe, S.K. (1985) Dry bean tannins: A review of nutritional implications. J. Amer. Oil Chem. Soc. 62: 541-549.

Richter, B.D. and Olds-Schneeman, B. (1987) Pancreatic response to long-term feeding of soy protein isolate, casein or egg white in rats. J. Nutr. 117: 247-252.

Ritter, M.A., Morr, C.V. and Thomas, R.L. (1987) In vitro digestibility of phytate-reduced and phenolics-reduced soy protein isolates. J. Food Sci. 52: 325-327, 341.

Roebuck, B.D. (1986) Enhancement of pancreatic carcinogenesis by raw soy protein isolate: quantitative rat model and nutritional considerations. Adv. Exp. Med. Biol. 199: 91-107.

Roebuck, B.D. (1987) Trypsin inhibitors: potential concern for humans? J. Nutr. 117: 398-400.

Roebuck, B.D., Kaplita, P.V, Edwards, B.R. Praissman, M. (1987) Effects of dietary fats and soybean protein on azaserine-induced pancreatic carcinogenesis and plasma cholecystokinin in the rat. Cancer Res. 47: 1313-1338.

Rogers, A.E. (1983) Influence of dietary content of lipids and lipotropic nutrients on chemical carcinogenesis in rats. Cancer Res. (Suppl.) 43: 2477s-2484s.

Rogers, A.E. and Newberne, P.M. (1975) Dietary effects on chemical carcinogenesis in animal models for colon and liver tumors. Cancer Res. 35: 3427-3431.

Rogers, A.E. and Newberne, P.M. (1980) Lipotrope deficiency in experimental carcinogenesis. Nutr. Cancer 2: 104-112.

Rothman, S.S. (1986) The biological functions and physiological effects of ingested inhibitors of digestive reactions. Chapter 2 in: Nutritional and Toxicological Significance of Enzyme Inhibitors in Foods, Friedman, M. (Ed.) Adv. Exp. Med. Biol. 199: 19-31.

Roy, D.M. and Schneeman, B.O. (1981) Effect of soy protein, casein and trypsin inhibitor on cholesterol, bile acids and pancreatic enzymes in mice. J. Nutr. 111: 878-885.

Rushmore, T.H., Lim, Y.P., Farber, E. and Ghoshal, A.K. (1984) Rapid lipid peroxidation in the nuclear fraction of rat liver induced by a diet deficient in choline and methionine. Cancer Lett. 24: 251-255.

Rushmore, T.H., Farber, E., Ghoshal, A.K., Parodi, S., Pala, M. and Taningher, M. (1986) A choline-devoid diet, carcinogenic in the rat, induces DNA damage and repair. Carcinogenesis 7: 1677-1680.

Ryan, C.A. (1973) Proteolytic enzymes and their inhibitors in plants. Ann. Rev. Plant Physiol. 24: 173-196.

Ryser, H.J.-P. (1971) Chemincal carcingenesis. New Eng. J. Med. 285: 721-734.

Sale, J.K., Goldberg, D.M., Fawcett, A.N., Wormsley, K.G. (1977) Chronic and acute studies indicating absence of exocrine pancreatic feedback inhibition in dogs. Digestion 15: 540-555.

Salman, A.J., Pubols, M.H., McGinnis, J. (1968) Chemical and microscopic nature of pancreata from chicks fed unheated soybean meal. Proc. Soc. Exp. Biol. Med. 128: 258-261.

Sambeth W., Nesheim, M.C. and Serafin, J.A. (1967) Separation of soybean whey into fractions with different biological activities for chicks and rats. J. Nutr. 92: 479-485.

Sarwar, G. and Botting, H.G. (1990) Rapid analysis of nutritionally important free amino acids in serum and organs (liver, brain and heart) by liquid chromatography of precolumn phenylisothiocyanate derivatives. J. Assoc. Off. Anal. Chem. 73: 470-475.

Sarwar, G. and Peace, R.W. (1986) Comparisons between true digestibility of total nitrogen and limiting amino acids in vegetable protein fed to rats. J. Nutr. 116: 1172-1184.

Sarwar, G., Botting, H.G. and Peace, R.W. (1989a) Amino acid rating method for evaluating protein adequacy of infant formulas. J. Assoc. Off. Anal. Chem. 72: 622-626.

Sarwar, G., Peace, R.W. and Botting, H.G. (1983) Validity of rat plasma amino acids in predicting first limiting amino acids in protein mixtures. Nutr. Rep. Int. 28: 613-620.

Sarwar, G., Peace, R.W. and Botting, H.G. (1989b) Differences in protein digestibility and quality of liquid concentrate and powder forms of milk-based infant formulas fed to rats. Am. J. Clin. Nutr. 49: 806-813.

Sastry, M.C.S. and Murray, D.R. (1987) The contribution of trypsin inhibitors to the nutritional value of chick pea seed protein. J. Sci. Food Agric. 40: 253-261.

Sathe, S.K. (1984) Enzyme inhibitors in dry beans. J. Amer. Oil Chem. Soc. 61: 691 (Abstr. 228).

Saxena, H.C., Jensen, L.S. and McGinnis, J. (1963a) Pancreatic hypertrophy and chick growth inhibition by soybean fractions devoid of trypsin inhibitor. Proc. Soc. Exp. Biol. Med. 112: 101-105.

Saxena, H.C., Jensen, L.S., McGinnis, J. and Lauber, J.K. (1963b) Histo-physiological studies on chick pancreas influenced by feeding raw soybean meal. Proc. Soc. Exp. Biol. Med. 112: 390-393.

Schneeman B.O. and Lyman, R.L. (1975) Factors involved in the intestinal feedback regulation of pancreatic enzyme secretion in the rat. Proc. Soc. Exp. Biol. Med. 148: 897-903.

Schneeman, B.O., Chang, I., Smith, L.B. and Lyman, R.L. (1977) Effect of dietary amino acids, casein and trypsin inhibitor on pancreatic protein secretion in rats. J. Nutr. 107: 281-288.

Scrimshaw, N.S., Wayler, A.H. , Murray, E., Steinke, F.H., Rand, W.M. and Young, V.R. (1983) Nitrogen balance response in young men given one of two isolated soy proteins or milk proteins. J. Nutr. 113: 2492-2497.

Sessa, D.J. (1984) Are toasted soybean flour proteins responsible for trypsin inhibitor activity? J. Am. Oil Chem. Soc. 61: 691.

Sessa, D.J. and Bietz, J.A. (1986) Toasted soybean flour components with trypsin inhibitor activity. J. Am. Oil Chem. Soc. 63: 784-788.

Sessa, D.J. and Ghantous, P.E. (1987) Chemical inactivation of soybean trypsin inhibitors. J. Am. Oil Chem. Soc. 64: 1682-1687.

Sgarbieri, V.C. and Whitaker, J.R. (1982) Physical, chemical and nutritional properties of common bean (Phaseolus) proteins. Adv. Food Res. 28: 93-165.

Shannon, B.M., Howe, J.M. and Clark, H.E. (1972) Interrelationships between methionine and cystine as reflected by growth, certain hepatic enzymes and liver composition of weanling rats. J. Nutr. 102: 557-562.

Shinozuka, H. and Farber, E. (1969) Reformation of nucleoli after ethionine-induced fragmentation in the absence of significant protein synthesis. J. Cell Biol. 41: 280-286.

Shinozuka, H., Lombardi, B., Sell, S. and Iammarino, R.M. (1978) Early histological and functional alterations of ethionine liver carcinogenesis in rats fed a choline deficient diet. Cancer Res. 38: 1092-1098.

Shivapurkar, N., and Poirier L.A. (1983) Tissue levels of S-adenosylmethionine and S-adenoslyhomocysteine in rats fed methyl-deficient, amino acid-defined diets for one to five weeks. Carcinogenesis 4: 1051-1057.

Shivapurkar, N., Wilson, M.J. and Poirier, L.A. (1984) Hypomethylation of DNA in ethionine-fed rats. Carcinogenesis 5: 898-982.

Shull, K.H., McConomy, J., Bogt, M., Costillo, A. and Farber, E. (1966) On the mechanism of induction of hepatic adenosine triphosphate deficiency by ethionine. J. Biol. Chem. 241: 5060-5070.

Sissons, J.W. (1982) Effects of soya-bean products on digestive processes in the gastrointestinal tract of preruminant calves. Proc. Nutr. Soc. 41: 53-61.

Sitren, H.S., Ahmed, E.M. and George, D.E. (1985) In vivo and in vitro assessment of antinutritional factors in peanut and soy. J. Food Sci. 50: 418-423.

Sklan, D., Budowski, P., Ascarelli, I. and Hurwitz, S. (1973) Lipid absorption and secretion in the chick: effect of raw soybean meal. J. Nutr. 103: 1299-1305.

Slaff, J., Jacobson, D., Tillman, C.R., Curington, C. and Toskes, P. (1984) Pancreatic-specific suppression of pancreatic exocrine secretion. Gastroenterol. 87: 44-52.

Smith, A.K. and Circle, S.J. (1972a) Historical background. Chapter 1 in: Soybeans: Chemistry and Technology Vol. 1, Proteins, Smith, A.K. and Circle, S.J. (Eds.) AVI pp 1-26. Publishing Co., Westport, Conn.

Smith, A.K. and Circle, S.J. (1972b) Protein products as food ingredients. Chapter 19 in: Soybeans: Chemistry and Technology Vol. 1, Proteins, Smith, A.K. and Circle, S.J. (Eds.) pp 339-388. AVI Publishing Co., Westport, Conn.

Smith, C., Van Megan, W., Twaalfhoven, L. and Hitchcock, C. (1980) The determination of trypsin inhibitor levels in foodstuffs. J. Sci. Food Agric. 31: 341-351.

Smith, J.T., Acuff, R.V., Bittle, J.B. and Gilbert, M. (1983) A metabolic comparison of cysteine and methionine supplements in the diet of a rat. J. Nutr. 113: 222-227.

Sosulski, F.W., Minja, L.A. and Christensen, D.A. (1988) Trypsin inhibitor and nutritive value in cereals. Plant Foods Hum. Nutr. 38: 23-34.

Snedecor, G.W. and Cochran, W.G. (1967) Statistical Methods 6th Ed., Iowa State University Press, Ames, Iowa,

Spangler, W.L., Gumbmann, M.R., Liener, I.E. and Rackis, J.J. (1985) The USDA trypsin inhibitor study. III. Sequential development of pancreatic pathology in rats. Qual. Plant. Foods Hum. Nutr. 35: 259-274.

Sri Kantha, S., Hettiarachchy, N.S. and Erdman, Jr., J.W. (1986) Nutrient, antinutrient contents, and solubility profiles of nitrogen, phytic acid, and selected minerals in winged bean flour. Cereal Chem. 63: 9-13.

Stipanuk, M.H. and Benevenga, N.J. (1977) Effect of cystine on the metabolism of methionine in rats. J. Nutr. 107: 1455-1467.

Stockland, W.L., Meade, R.J., Wass, D.F. and Sowers, J.E. (1973) Influence of levels of methionine and cystine on the total sulfur amino acid requirement of the growing rat. J. Anim. Sci. 36: 526-530.

Struthers, B.J. and MacDonald, J.R. (1983) Comparative inhibition of trypsins from several species by soybean trypsin inhibitors. J. Nutr. 113: 800-804.

Struthers, B.J., MacDonald, J.R., Dahlgren, R.R. and Hopkins, D.T. (1983a) Effects on the monkey, pig and rat pancreas of soy products with varying levels of trypsin inhibitor and comparison with the administration of cholecystokinin. J. Nutr. 113: 86-97.

Struthers, B.J., MacDonald, J.R., Prescher, E.E. and Hopkins, D.T. (1983b) Influence of several plant and animal proteins on rat pancreas. J. Nutr. 113: 1503-1512.

Swaim, M.W. and Pizzo, S.V. (1988) Methionine sulfoxide and the oxidative regulation of plasma proteinase inhibitors. J. Leukocyte Biol. 43: 365-379.

Tan-Wilson, A.L. and Wilson, K.A. (1986) Relevance of multiple soybean trypsin inhibitor forms to nutritional quality. Adv. Exp. Med. Biol. 199: 391-412.

Tan-Wilson, A.L., Chen, J.C., Duggan, M.C., Chapman, C, Obach, R.S. and Wilson, K.A. (1987) Soybean Bowman-Birk trypsin isoinhibitors: Classification and report of a glycine-rich trypsin inhibitor class. J. Agr. Food Chem. 35: 974-981.

Tateishi, H. Higashi, T. Naruse, A., Nakashima, K., Shiozaki, H and Sakamoto, Y. (1977) Rat liver glutathione: possible role as a reservoir of cysteine. J. Nutr. 107: 51-60.

Tatematsu, M., Nagamine, Y. and Farber, E. (1983) Redifferentiation as a basis for remodelling of carcinogen-induced hepatocyte nodules to normal appearing liver. Cancer Res. 43: 5049-5058.

Temler, R.S. (1980) Alterations in the pancreas of rats fed on different levels of soya flour and casein. Int. J. Vitam. Nutr. Res. 50: 212-213.

Temler, R.S., Dormond, C.A., Simon, E., Morel, B. and Mettraux, C. (1984a) Response of rat pancreatic proteases to dietary proteins, their hydrolysates and soybean trypsin inhibitor. J. Nutr. 114: 270-278.

Temler, R.S., Dormond, C.A., Simon, E. and Morel, B. (1984b) The effect of feeding soybean trypsin inhibitor and repeated injections of cholecystokinin on rat pancreas. J. Nutr. 114: 1083-1091.

Theuer, R.C. and Sarett, P. (1970) Nutritional adequacy of soy isolate infant formulas in rats: choline. J. Agr. Food Chem. 18: 913-916.

Tietz, N.W. (1983) General clinical tests. Section 1 in : Clinical Guide to Laboratory Tests, Tietz, N.W. (Ed.) pp1-559. W.B. Saunders, London.

Troll, W., Wiesner, R., Shellabarger, C.J., Holtzman, S. and Stone, J.P. (1980) Soybean diet lowers breast tumor incidence in irradiated rats. Carcinogenesis (Lond.) 1: 469-472.

Troll, W., Frenkel, K. and Weisner, R. (1986) Protease inhibitors: their role as modifiers of carcinogenic processes. Adv. Exp. Med. Biol. 199: 153-165.

Turner, R.H. and Liener, I.E. (1975) The effect of selective removal of hemagglutinins on the nutritive value of soybeans. J. Agric. Food Chem. 23: 484-487.

Valdebouze, P., Bergeron, E., Gaborit, T. and Delort-Laval, J. (1980) Content and distribution of trypsin inhibitors and hemagglutinins in some legume seeds. Can. J. Plant Sci. 60: 695-701.

Vaughan, D.A., Womack, M. and McClain, P.E. (1977) Plasma free amino acid levels in human subjects after meals containing lactalbumin, heated lactalbumin or no protein. Am. J. Clin. Nutr. 30: 1709-1712.

Vince, A.J., McNeil, N.I., Wager, J.D. and Wrong, O.M. (1990) The effect of lactulose, pectin, arabinogalactan and cellulose on the production of organic acids and metabolism of ammonia by intestinal bacteria in a faecal incubation system. Br.J. Nutr. 63: 17-26.

Waggle, D.H., Steinke, F.H. and Shen, J.L. (1990) Isolated soy proteins. Chapter 4 in: Legumes Chemistry, Technology and Human Nutrition Matthews, R.H. (Ed.) pp 99-138. Marcel Dekker Inc. N.Y., Basel.

Wang, C.R. and Chang, S.K.C. (1988) Effect of selected canning methods on trypsin inhibitor activity, sterilization value, and firmness of canned navy beans. J. Agric. Food Chem. 36: 1015-1018.

Wang, H.L. (1984) Tofu and tempeh as potential protein sources in the Western diet. JAOCS 61: 528-534.

Wang, H.L., Swain, E.W., Wallen, L.L. and Hesseltine, E.W. (1975) Free fatty acids as antitryptic factor in soybeans formulated by Rhizopus oligosporus. J. Nutr. 105: 1351-1355.

Waxman, D.J. (1990) Glutathione-S-transferases: role in alkylating agent resistance and possible target for modulation chemotherapy--a review. Cancer Res. 50: 6449-6454.

Weder, J.K.P. (1986) Inhibition of human proteinases by grain legumes. Adv. Exp. Med. Biol. 199: 239-279.

Weder, J.K.P. and Mueller, R. (1989) Reaction of lentil proteinase inhibitors with human and bovine trypsin and chymotrypsin. J. Food Biochem. 13: 81-103.

Weder, J.K.P., Mueller, R., Ildefonso, M.C. and Hegarty, M.P. (1985) Proteinase inhibitor activities and electrophoretic inhibitor patterns of lentils. Lens Newsletter 12: 29-32.

Westfall, F.J. and Hauge, S.M. (1947) The protein efficiency of soybean flour as related to its trypsin inhibitor content. Fed. Proc. 6: 432-436.

Westfall, F.J., Bosshardt, D.K. and Barnes, R.H.. (1948) Influence of crude trypsin inhibitor on utilisation of hydrolysed protein. Proc. Soc. Exp. Biol. Med. 68: 498-500.

Wieczorek, M., Otlewski, J., Cook, J., Parks, K., Leluk, J., Wilimowska-Pelc, A., Polanowski, A., Wilusz, T. and Laskowski, Jr., M. (1985) The squash family of serine proteinase inhibitors. Amino acid sequences and association equilibrium constants of inhibitors from squash, summer squash, zucchini, and cucumber seeds. Biochem. Biophys. Res. Comm. 126: 646-652.

Wilson, B.J. (1980) The release of proteinase inhibitors from legume seeds during germination. Phytochem. 19: 2517-2519.

Wilson, B.J., McNab, J.M. and Bentley, H. (1972) Trypsin inhibitor activity in the field bean (Vicia faba L.). J. Sci. Fd. Agric. 23: 679-684.

Wilson, K.A., Papastoitsis, G., Hartl, P., and Tan-Wilson, A.L. (1988) Survey of the proteolytic activities degrading the Kunitz trypsin inhibitor and glycinin in germinating soybeans (Glycine max). Plant Physiol. 88: 355-360.

Wink, D. (1985) Getting through the maze of infant formulas. Am. J. Nursing 85: 388-392.

Wolf, W.J. and Cowan, J.C. (1975) Soybeans As A Food Source Revised edition. CRC Press, Cleveland, Ohio.

Wormsley, K.G. (1988) Trypsin inhibitors: potential concern for humans? J. Nutr. 118:134-136.

Xavier-Filho, J. and Campos, F.A.P. (1989) Protease inhibitors. Chapter 1 in Toxicants of Plant Origin Vol. III Proteins and Amino Acids. Cheeke P.R. (Ed.) pp 1-27. CRC Press Inc. Boca Raton, Florida.

Yamaguchi T.m Tabata, K. and Johnson, L.R. (1985) Effect of proglumide on rat pancreatic growth. Am. J. Physiol. 249: G294-G298.

Yanatori, Y. and Fujita, T. (1976) Hypertrophy and hyperplasia in endocrine and exocrine pancreas of rats fed soybean trypsin-inhibitor or repeatedly injected with pancreozymin. Arch. Hist. J. 39: 67-78.

Yavelow, J., Finlay, T.H., Kennedy, A.R. and Troll, W. (1983) Bowman-Birk soybean protease inhibitor as an anticarcinogen. Cancer Res. (Suppl.) 43: 2454s-2459s.

Yen, J.T., Hymowitz, T. and Jensen, A.H. (1974) Effects of soybeans of different trypsin-inhibitor activities on performance of growing swine. J. Anim. Sci. 38: 304-309.

Yen, J.T., Jensen, A.H. and Simon, J. (1977) Effect of dietary raw soybean and soybean trypsin inhibitor on trypsin and chymotrypsin activities in the pancreas and in small intestinal juice of growing swine. J.Nutr. 107: 156-165.

Yokoyama, S., Sells, M.A., Reddy, T.V. and Lombardi, B. (1985) Hepatocarcinogenic and promoting action of a choline-devoid diet in the rat. Cancer Res. 45: 2834-2842.

Young, V.R., Tontisirin, K., Ozalp, I, Lakshmanan, F., Scrimshaw, N.S. (1972) PAA response curve and amino acid requirements in young rats: valine and lysine. J. Nutr. 102: 1159-1165.

Zeisel, S.H., Zola, T., DaCosta, K.-A. and Pomfret, E.A. (1989) Effect of choline deficiency on S-adenosylmethionine and methionine concentrations in rat liver. Biochem. J. 259: 725-729.

Zlotkin, S.H. and Anderson, G.H. (1982) The development of cystathionase activity during the first year of life. Pediatr. Res. 16: 65-68.

APPENDIX A

OPERATING PARAMETERS FOR ANALYSIS OF AMINO ACIDS

This appendix describes the system and conditions used for analysis of the amino acids in the protein sources used in the various experiments. A list of amino acid composition for each protein source is shown on the next page.

a) 121 MB Amino Acid Analyzer, Model 126 Data System, single column (0.28 x 23 cm) system, AA-10 resin, sodium citrate buffers and ninhydrin solutions available from Beckman Instruments (Spinco Division, 1117 California Ave., Palo Alto, CA).

b) Flow rates:

Buffer solutions	8.8 mL/hr
Ninhydrin solution	4.4 mL/hr

c) Buffers used:

1. pH 3.25, 0.16 N sodium citrate buffer
2. pH 3.28, 0.20 N sodiun citrate buffer
3. pH 3.90, 0.35 N sodium citrate buffer
4. pH 4.00. 0.44 N sodium citrate buffer
5. Buffer 4:Buffer 6 (8:13 vol/vol)
6. pH 6.40, 1.0 N sodium citrate buffer
7. 0.2 N NaOH regeneration solution

d) System Operating Conditions:

Buffer	Buffer Run Times	Total Elapsed Time	Temperature
1	26 mins	26 mins	33 °C
2	19 mins	45 mins	51 °C
3	3 mins	48 mins	51 °C
4	13 mins	61 mins	51 °C
5	40 mins	101 mins	51 °C
6	18 mins	119 mins	51 °C
7	4 mins	123 mins	51 °C
1	18 mins	141 mins	51 °C

e) Connection of the two independent four-way buffer exchange values in series in front of the buffer pump permitted up to 7 buffer changes including the NaOH regeneration step.

TABLE A.1 Amino acid composition[1] of the protein sources used in animal experiments

L-amino acid	Protein Source		
	ANRC Casein[2]	Soy protein isolate[3]	Soybean[4]
Asp	487	698	695
Thr	296	239	242
Ser	395	323	323
Pro	741	308	301
Glu	1525	1137	1086
Gly	127	255	258
Ala	208	263	257
Val	442	303	288
Cys	26	68	84
Met	194	78	85
Ile	350	290	292
Leu	665	500	472
Tyr	389	241	230
Phe	360	316	299
NH_2	114	97	94
Lys	546	376	374
His	194	160	160
Try	72	83	75
Arg	252	510	511

[1] mg/g Nitrogen, based upon amino acid analyses of acid and base hydrolysates of protein sources.
[2] ANRC Casein: Animal Nutrition Research Council Casein, Humko Sheffield, Kraft Co., Norwich, NY.
[3] Soy protein isolate: Supro 710, Ralston Purina Co., St. Louis, MO.
[4] Soybean: raw, ground Maple Arrow Soybeans, Hardy Seeds Ltd., Inkerman, Ont.