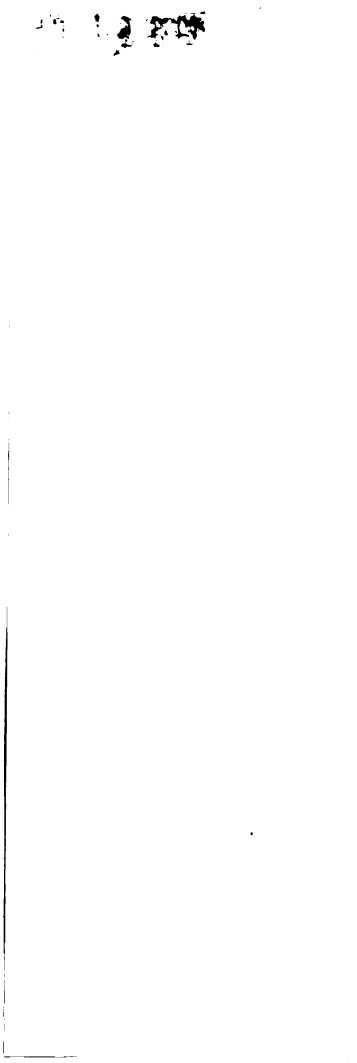

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ABSTRACT

FACTORS IN SOYBEANS INFLUENCING GROWTH, PANCREATIC FUNCTION AND DIGESTION

By

David J. Schingoethe

The effect of age on pancreas size and proteolytic enzyme content in bull calves from birth to one year of age was studied under normal dietary conditions. Pancreas weight, trypsin and chymotrypsin activities increased directly with body weight over the age span studied. The new born calf was the only age group which indicated any marked differences from the mean of all age groups. Pancreas weight and trypsin content were slightly less at this age while chymotrypsin content per mg of pancreas tissue was higher than at all other ages.

Weanling rats were used to compare milk protein with soybean protein sources either containing or lacking soybean trypsin inhibitor (SBTI) to study factors influencing growth, pancreatic function and intestinal protein digestion. Diets containing SBTI caused pancreatic enlargement with corresponding increases in trypsin and chymotrypsin activities. Enzyme activities per mg of pancreas tissue remained constant over all dietary treatments. These same SBTI-containing diets caused increased chymotrypsin

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activity and stability, decreased trypsin stability, but did not change free trypsin activity in the intestinal contents. Intestinal protein digestion, as measured by an in vitro system, was not impaired in the intestinal contents of SBTI-fed rats. Growth rates were depressed by only two of the four SBTI diets indicating that the growth depression exerted by raw or minimally processed soybean products is not caused by SBTI and apparently occurs by some mechanism other than by interference with protein digestion.

Raw (unheated) soybean meal was subjected to numerous physical and chemical treatments in efforts to isolate a growth inhibitor fraction which was free of SBTI activity. Each treatment fraction was added to the diet of growing mice and growth inhibition determined by comparing their growth rates to growth rates achieved on an autoclaved soybean meal diet. A small molecular weight growth inhibitor was separated from SBTI by ion exclusion chromatography on a Sephadex G-50 column and partially characterized. This growth inhibitor decreased weight gains and feed efficiencies of mice without causing pancreatic enlargement. Evidence of the small size of this growth inhibitor was retardation on a Sephadex G-25 column, removal by dialysis and lack of detection by polyacrylamide-gel electrophoresis. Movement toward the cathode under high voltage electrophoresis at pH 3.5 and apparent adsorption on DEAE-cellulose indicated a positively charged compound at that pH.

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Other factors in soybeans may also inhibit growth. The water-insoluble residue accounted for about 40% of the growth inhibitor activity of raw soybean meal. This growth inhibition could not be removed or destroyed by gastro-intestinal enzyme digestions or by several other solvents tested. Fractions containing SBTI generally caused pancreatic enlargement and usually caused some growth inhibition.

FACTORS IN SOYBEANS INFLUENCING GROWTH, PANCREATIC FUNCTION AND DIGESTION

By

David J. Schingoethe

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AUTOBIOGRAPHY

I was born February 15, 1942 in Aurora, Illinois, and was raised with two younger brothers on my parents dairy and grain farm in northeastern Illinois. I graduated from Kaneland High School, Maple Park, Illinois in June of 1960. I received the Bachelor of Science degree in Agricultural Science from the University of Illinois in June, 1964, and the Master of Science degree from the Dairy Science Department at the same institution in October, 1965. Research for the M.S. degree was conducted in dairy biochemistry under the guidance of Dr. B. L. Larson. I enrolled as a graduate student in the Department of Dairy and Institute of Nutrition at Michigan State University in September, 1965.

I am a member of the American Dairy Science Association, the American Society of Animal Science, Sigma Xi, Alpha Zeta and Gamma Sigma Delta. I am married to the former Darlene Wennlund, a University of Illinois graduate.

THE

TABLE OF CONTENTS

THE

LIST OF TABLES

Table

29.

30.

LIST OF FIGURES

LIST OF APPENDIX TABLES

CHAPTER I

INTRODUCTION

The relatively low cost of many plant source ingredients has stimulated interest and research concerning their possible use in milk substitutes for calves as well as humans. Soybeans are a principle source of such ingredients. However, soybeans contain several substances which impair growth, cause pancreatic enlargement, inhibit intestinal protein digestion by pancreatic enzymes, and may interfere with intestinal absorption as well as metabolic functions of several body organs. Fortunately, these undesirable factors are heat labile and thus can be readily destroyed or inactivated by heat applied during extraction or processing. However, such heating reduces the solubility of the product, which makes the product unacceptable for use in milk substitutes. This is because materials must be soluble or easily suspended to be acceptable in a liquid milk substitute preparation. Thus, the undesirable factors in soybeans must be more adequately characterized so that a more desirable method of destroying or removing them can be developed.

The purpose of the research reported herein was to characterize factors in soybeans which depressed growth,

and determine if these same factors were responsible for the changes in pancreatic output and function. In addition, the effect of age on pancreas size and proteolytic enzyme content in bull calves from birth to one year of age was studied under normal dietary conditions.

CHAPTER II

LITERATURE REVIEW

A. Pancreas Size and Enzyme Activity as Affected by Species, Age, and Diet.

The pancreas, in addition to being an endocrine organ, is a major source of enzymes for digesting dietary nutrients. The pancreatic excretory enzymes include the proteases trypsin, chromatrypsin, carboxypeptidases A and B, and elastase, the nucleases-ribonuclease and deoxyribonuclease, the carbohydrase amylase and the fat digesting lipase. The relative proportions of these enzymes in pancreatic juice are shown in Table 1. The proteases are synthesized, stored and secreted in the inactive zymogen forms of trypsinogen, chymotrypsinogen and procarboxypeptidase A and B. Activation is initiated in the small intestine by enterokinase. Pancreatectomy results in decreased amino acid and fat absorption (27). This leads to a subsequent loss of body weight and blood lipid concentration together with the development of a fatty liver with impaired function.

The relative importance of the pancreas may be different in the mature ruminant than in the immature ruminant or monogastric. This is because of the more constant

TABLE 1.--Relative proportion of components of bovine pancreatic juice (72).

Enzyme or Proenzyme	Per cent of total Protein
Proteolytic	
Trypsinogen	14
α Chymotrypsinogen	16
β Chymotrypsinogen	16
Procarboxypeptidase A	19
Procarboxypeptidase B	7
Nucleolytic	
Ribonuclease	2.4
Deoxyribonuclease	1.4
Amylase	<2
Lipase	very low
Unidentified	>10

intestinal flow and the pregastric digestion in the rumen. Another consideration is the relationship between pancreas size and body weight. Brody (23) reported that the size of several internal body organs increased with the 0.70 to 0.80 power of body weight, but no data were presented for the pancreas.

Prenatal Development

Proteolytic activity or zymogen granules were detected in the fetal pancreas after 13 days of gestation in the mouse (104) and chick (99), 50 to 53 days in the pig (125), and 5 to 6 months of pregnancy in the meconium of humans (87). The cytoplasm of mice fetal pancreases was completely filled with zymogen granules by 18 days (104), while maximum amount of proteolytic enzymes occurred at 22 days of development (1 day-old chick) in the chick pancreas (99), and at 60 to 62 days in the pig embryo (125).

Postnatal Development

Levels of pancreatic protease, lipase, and amylase are low at birth in most species (23, 32, 64, 65, 87, 99, 104, 112, 121, 125, 151). Pancreatic and intestinal enzyme activities, of calves remained fairly constant up to 6 weeks of age (32, 49), although a threefold increase in pancreatic amylase and protease from 7 to 44 days of age was noted by one group of investigators (64). Amylase concentration in pig pancreases increases twentyfold from

1 to 38 days of age (65). Rat pancreatic amylase developed most rapidly during the period from 15 to 20 days of age (112).

Trypsin and chymotrypsin content of human pancreases did not increase until about one month post partum (87), resulting in a relatively small increase in proteolytic activity of duodenal contents during this interval (75). Concentration of proteolytic enzymes in the baby pig pancreas did not increase with age (86), suggesting that pancreas growth may control total enzyme secretion.

A limited amount of data on two calves at each of several ages from 1 to 44 days of age indicated that pancreas size ranged from 0.06 to 0.10% of body weight and appeared to increase directly with body weight (64). Table 2 summarizes pancreas size data from several species.

Pancreatic juice secretion in calves increased from 125 ml/24 hr at 3 or 7 days of age to 1300 ml/24 hr at 6 months of age (101). Total trypsin output increased slightly from 3 or 7 days of age to 21 days of age (50).

The ratios of chymotrypsin-to-trypsin activities in pancreatic tissue and juice, and intestinal contents showed marked species difference (46, p. 26). Some of the differences are now known to be due to enzyme substrates used in the assay procedure, other assay conditions (48) and dietary factors (135). Ratios varied from as low as

TABLE 2.--Pancreatic size in several vertebrate species, calculated from published data.

Species	Sex	Body wt. (kg)	Pancreas Size (g/kg Body wt.)	Reference
Bat	male	0.00543	2.76	(148) compiled
	female	0.00541	9.24	by (57)
Shrew	male	0.00754	13.26	"
	female	0.00757	14.72	"
Lizard	male	0.0126	1.66	"
	female	0.0128	4.06	"
Mouse	male	0.0141	6.30	"
	female	0.0159	8.57	"
Salamander	male	0.0257	0.82	"
	female	0.0242	1.98	"
Frog	male	0.0638	1.44	"
	female	0.0555	1.28	"
Rat (30-50 days)	male	0.03-0.10	7.19	"
	female	0.03-0.10	7.60	"
Chick (12-14 days)		0.205	4.39	(5)
Hedgehog	male	0.258	17.55	(148) compiled
	female	0.389	8.48	by (57)
Cat	male	2.83	2.08	"
	female	2.00	2.65	"
Dog	male	4.86	3.30	"
	female	4.99	4.02	"
	Both sexes	13.6	1.85	(28)
Pig		20[a]	2.10	(62)
		100[a]	1.55	
Humans	male	56-95	1.16 $\pm$ .22[b]	(129)
	female	48-95	1.16 $\pm$ .32[b]	
Calf	male	39-62	0.63	(46)

[a]Estimated body weight.

[b]Mean $\pm$ standard deviation.

0.14-0.17 in rabbit pancreas juice (122), using ATEE[a] and TAME[b] for chymotrypsin and trypsin substrates, respectively, to as high as 19.2 in bovine pancreas juice, using ATEE and BAEE[c] as substrates (72). Most studies place the chymotrypsin-to-trypsin ratios in the range of 1 to 3; however, ratios of less than one were consistently observed in bovine pancreases (49) and pancreatic juice (50, 130), while ratios of greater than one were observed in intestinal contents of rats and chicks (47).

Dietary Adaptation

The pancreas adapts to specific dietary nutrients by altering the proportions of several pancreatic enzymes (31). High starch diets elevated amylase and reduced protease, while high protein diets induced the opposite changes in rat pancreatic tissue or its exocrine secretions (6, 7, 52, 63, 100, 118). Amylase represented 25% of total pancreatic proteins on a high starch diet but only 8% on a high protein diet (100). Pancreatic lipase showed no adaptation to dietary fat, but increased on high protein diets (52). Alterations of pancreatic exocrine enzymes were detected one day after a dietary change, were rapid from 3 to 5 days, and were complete by 7 (137) or 8 days (6, 7). The total pancreatic protein output was

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[a]ATEE, acetyl tryosine ethyl ester.

[b]TAME, toluene arginine methyl ester.

[c]BAEE, benzoyl arginine ethyl ester.

not appreciably altered by dietary changes (7). Chromatography of pancreatic juice on DEAE cellulose proved that diet modified the amount and not the activity of these enzymes (7).

Several studies indicated that dietary alterations modify chymotrypsinogen systhesis to a greater extent than trypsinogen synthesis (6, 7, 119, 135). Relatively more of the pancreas amino acid supply was diverted to chymotrypsinogen and less to amylase when whole-egg protein was fed (135). The reverse occurred when casein was given. This may be due to the lower methionine content of casein since the pancreases of rats fed a methionine-deficient diet contained normal amounts of amylase, reduced amounts of chymotrypsin and no trypsin (145). Snook (135, 136) observed ratios of chymotrypsin-to-trypsin activities in rat pancreases of 1.64 on a protein-free diet, 1.93 to 2.04 on a casein-protein diet and 2.74 to 2.86 on a whole-egg protein diet. When rats were chan changed from a high-starch to a high-protein diet, incorporation of C^{14}-valine into chymotrypsinogen rose from 88 to 266 cpm whereas C^{14} activity in trypsinogen increased only from 87 to 116 cpm (119).

B. Soyflour in Calf Milk Replacers

The increased use of plant proteins in calf milk replacers would appear advantageous for economic reasons. Although some (139, 140, 141) obtained acceptable growth

rates in calves fed a mixture of plant and milk protein, growth from plant protein alone usually was unsatisfactory (40, 108, 109, 140). Forty per cent raw soybean meal (RSBM) in calf milk replacer resulted in 100% mortality by 27 to 58 days of age (151). Adding proteolytic enzymes to the replacer did not improve the utilization of soy protein (83, 108). Predigesting fully cooked soyflour with various proteolytic enzyme preparations did not improve its nutritive value in calf milk replacers, but acid digestion at pH 4.0 for five hours at 37°C did bring its nutritive value up to almost that of the positive control milk protein group (26).

Gorrill and Thomas (49) and Gorrill et al. (50) studied the effects of soybean and milk protein diets on pancreas proteolytic enzyme content and secretion, and on intestinal proteolytic activity in efforts to determine the mechanism whereby soy protein causes poor growth in calves. Pancreatic trypsin and chymotrypsin secretion, as well as activities in the pancreas and intestinal contents were suppressed in calves fed a soyflour milk replacer containing relatively high levels of soybean trypsin inhibitor (SBTI). This response did not occur in calves fed an all-milk replacer nor in those fed a soy-protein-concentrate replacer containing very low levels of SBTI. Since calves lost weight on soyflour diets containing significant levels of SBTI (26, 49) they speculated

that SBTI may be the cause of poor growth rates presumably via its adverse affect on pancreatic response. However, since these calves also had considerable diarrhea (49), one cannot unequivocally say that SBTI is the causative factor of the observed pancreatic and growth responses.

C. Raw Soybeans and Growth Inhibition

Osborne and Mendel (110) in 1917 reported the improved growth-promoting effect of cooked versus raw soybeans. Despite the numerous studies and improved technology of the past 50 years, the explanation for this observation is still unclear. Several factors have been implicated as causing the growth inhibition associated with raw soybeans and these will be discussed in the following pages.

Trypsin and Chymotrypsin Inhibitors

Table 3 summarizes some of the physical and chemical properties of several trypsin inhibitors isolated from raw soybeans. Kunitz (77) first crystallized a soybean trypsin inhibitor (CSBTI) in 1946. This inhibitor has been most widely studied and is the one generally available commercially. CSBTI is the only major soybean trypsin inhibitor to contain significant amounts of tryptophan (15). F_1 and F_3 inhibitors, which were isolated from a commercial crude soybean trypsin inhibitor, also contain tryptophan but F_3 is devoid of tryosine (39). Amino acid analyses also indicate that inhibitors AA, 1.9S, F_1 and F_3 contain

TABLE 3.--Physical and chemical properties of soybean trypsin inhibitors.

Property	CSBTI	A_1	B_1	B_2	AA	1.9S	F_1	F_3
Molecular weight	22,700	14,300	---	---	24,000	16,400	18,300	23,400
Electrophoretic mobility $X10^5$ (cm^2 $volt^{-1}$ sec^{-1})	-8.0	-7.4	-4.5	-5.3	---	-8.45	---	---
Sedimentation constant, $S_{20}W$	2.30	1.80	4.07	4.62	2.30	1.90	---	---
Partial specific volume (ml/g)	0.745	0.736	---	---	---	0.69	---	---
Extinction coefficient, $E.^{1\%}_{1\ cm}$ at 280 mu	0.900	0.942	---	---	0.48	0.44	0.716	0.634
Nitrogen content (%)	16.34	14.96	---	---	15.90	15.28	---	---
N-terminal amino acid	Asp.	Asp.	---	---	---	Asp.	---	---
C-terminal amino acid	leu.	---	---	---	---	---	---	---
Specific activity (mg trypsin inhibited/mg inhibitor)	1.0	1.60	2.0	1.8	---	1.8	---	---
Reference	(77)	(114, 116)	(114)	(114)	(15)	(154)	(39)	(39)

unusually high levels of half cystine (11, 39, 70, 153, 154). They contain 34, 26, 14, and 12 half cystine residues per molecule, respectively, while CSBTI contains only four. Rackis et al. (114, 116) also isolated a trypsin inhibitor designated $SBTIA_2$ which is identical to Kunitz's (77) CSBTI.

Most trypsin inhibitors also inhibit chymotrypsin, but to varying degrees (15, 39, 44, 114, 116, 154). Inhibitors AA (15) and 1.9S (154) are fairly potent chymotrypsin inhibitors, while other trypsin inhibitors are less inhibitory towards chymotrypsin. Gertler et al. (44) determined that AA contained 8.0 trypsin-inhibitor units (TIU) and 10.0 chymotrypsin-inhibitor units (ChIU) per milligram of inhibitor as opposed to 6.0 TIU and 0.75 ChIU for CSBTI. The order of trypsin inhibitor potency of soy fractions characterized to date is: CSBTI = A_1 = AA = 1.9S > F_1 > F_3 (39). The approximate order for chymotrypsin is: 1.9S $\geq$ AA $\geq$ A_1 > CSBTI > F_1 >> F_3 (39).

Most presently known soybean trypsin inhibitors stoichiometrically inhibit trypsin by forming a virtually inseparable complex (51, 82). Sealock and Laskowski, Jr. (132) showed that this was due to a close cystine disulfide bridge across the active arginine residue which did not allow the trypsin inhibitor molecule to open and release the trypsin once the arginyl residue had been hydrolyzed. Inhibitor AA is noncompetitive (10) while

CSBTI is competitive (51) to the proteolytic and esterolytic activities of trypsin and chymotrypsin on casein, BAEE, and ATEE, respectively.

The finding of a trypsin inhibitor in raw soybeans by Ham and Sandstedt (54) in 1944 offered a probable explanation to the mechanism whereby heat treatment improved the soybean's nutritional value. Trypsin inhibitor activity is destroyed by autoclaving (54, 150). Further evidence to support this explanation came from observations that the addition of crude trypsin inhibitor to diets containing heated raw soybean mean (HRSBM) reduced the growth rate of chicks (55) and rats (74). Because of these early results, trypsin inhibitors have since received the bulk of attention in attempts to further elucidate the cause of growth depression by raw soybeans.

Adding CSBTI to chick (42, 44) and rat (44, 53) diets depressed growth but not to the extent of the depression caused by RSBM diets. Garlich and Neshein (42) batch separated the soybean whey fraction obtained by the procedures of Rackis et al. (115) into two fractions, one high in trypsin-inhibiting activity and the other high in hemagglutinating activity. Neither fraction when fed alone affected growth rate to the same extent as a combination of the two fractions. The whey fraction contains most of the soybean trypsin inhibitors and hemagglutinins, along with other unidentified components (35, 116). Geratz (43)

caused growth depression in rats by adding p-aminobenzamidine, a potent trypsin inhibitor,to their drinking water, but most of the growth depression was attributed to decreased feed intake with only a moderate decrease in efficiency of gain being observed.

Some suggested that the growth depression associated with trypsin inhibitor could be overcome by adding trypsin to the ration. However, adding crude or crystalline trypsin to a raw soybean meal diet for chicks (22) and rats (20) failed to remove its growth-depressing properties.

If the growth depression observed when animals are fed unheated soybean meal is due to interference with intestinal tract enzymatic digestion, feeding amino acids instead of intact protein should alleviate the problem. Desikachar and De (30) obtained poor biological values when they fed a papain digest of RSBM to which had been added an aqueous extract of RSBM containing the trypsin inhibitor. Similar results were obtained when crude trypsin inhibitor was added to a casein digest diet for mice (149), or to amino acid diets for chicks (60) and rats (73). Thus, the growth depression mechanism appears to be something other than an inhibition of intestinal enzymatic activity. Since only crude trypsin inhibitor preparations were used in all of these studies the possibility still remains that the causative factor was not trypsin inhibitor.

The compensatory capacity of the animal also indicates trypsin inhibition is probably not the growth depressant factor in raw soybeans. Even if the soybean trypsin inhibitor tied up more than half of the trypsin in the intestinal tract, the pancreas should be able to compensate for it by secreting extra enzymes. Scow (131) showed no reduction in nitrogen digestion and absorption as measured by fecal excretion in rats having 95% of their pancreatic tissue removed. Reduction in protein digestion was observed only when 99.5% of the pancreatic tissue was removed. Khayambashi and Lyman (73) noted more intestinal protease and more TCA-insoluble nitrogen in the intestinal contents of rats fed SBTI. However, somewhat contradictory to the above is the fact that intestinal proteolysis was almost completely inhibited in chicks up to 3 weeks old fed a raw soybean diet, but increased from the fourth week, reaching normal at six weeks of age (2).

Studies with germinated soybeans give additional evidence that trypsin inhibitor is not the growth depressant factor. Rats fed germinated seeds grew much better than those fed raw soybeans and had protein efficiency ratios almost equal to those fed autoclaved soybean meal (37). This occurred even though the trypsin inhibitor concentrations remained the same as in the raw soybeans (30).

Because soybeans are more resistant to insect attack than many of the seed grains, insects have been used in some soybean proteolytic inhibitor studies (10, 95). The soybean protein fraction designated C_1 inhibits growth and in vitro proteolytic activity in Tribolium larvae, inhibits trypsin and chymotrypsin, and possesses amylase activity. However, the in vitro proteolytic activity of Tribolium larvae was not affected by the trypsin inhibitors CSBTI, AA, lima bean trypsin inhibitor or ovumucoid (16). This indicated that the trypsin-inhibiting component of the C_1 protein fraction was not responsible for inhibiting the larval proteinase. Chromatographic separation of the C_1 fraction on a calcium phosphate (hydroxyapatite) column showed that the Tribolium proteinase inhibitor is a distinct inhibitor that is free of trypsin- and chymotrypsin-inhibitor and free of amylase (14).

Hemagglutinin

In addition to trypsin inhibitors, the hemagglutinin in raw soybeans has been implicated as a growth inhibitor. The presence of hemagglutinating agents in plants was recognized in the 1880's (91). Several groups showed that extracts from various seeds agglutinate red blood cells from some animal species but not the cells from other species (9, 81, 93).

Studies concerning a possible role of hemagglutinin in explaining the mechanism of growth depression caused

by feeding RSBM were initiated by Liener et al. (92) when they had difficulty explaining a growth depression resulting from feeding crude trypsin inhibitor to a diet containing a protein hydrolysate. Liener and Pallansch (93) isolated a homogeneous protein in 1952 high in hemagglutinin activity. Further purification indicated it has a molecular weight of 96,000 and contains 6-10% glucosamine (146). Lis et al. (96) found four distinct hemagglutinins in soybean oil meal which were separable on DEAE-cellulose columns. The most abundant hemagglutinin is identical with that previously described by Liener and co-workers (93, 146). All four are glycoproteins containing mannose and glucosamine.

Hemagglutinins do not appear to be closely associated with the growth inhibiting properties of soybeans. Liener (88) and Stead et al. (138) reported that intraperitoneal injections of hemagglutinin preparations were lethal to young rats. However, this information is of questionable physiological significance for two reasons: (1) hemagglutinin is readily inactivated by peptic digestion, even when as few as 12% of its peptide bonds are split (13, 90); therefore, it should be either completely or almost completely inactivated before entering the small intestine; and (2) even if hemagglutinins did survive gastric digestion, an intact protein of 96,000 molecular weight would not likely be absorbed from the gut. Thus, Liener (89)

made an optimistic estimation that soyin, the name originally given to soybean hemagglutinin, may account for 50% of the growth inhibition of raw soybean meal. In a later study (146) he found that it decreased rat growth only 25% below that of the positive control group with essentially all of this decrease being attributed to decreased feed intake. Birk and Gertler (13) found that the fraction containing about 50% of the original trypsin inhibitor and about all of the hemagglutinin from raw soybean meal only slightly inhibited the growth rates of rats, chicks, and the larvae of Tribolium castaneum. Also, during the purification process of soybean hemagglutinin (93), there was no correlation between toxicity of the fractions and their hemagglutinating activities. The progressive increase in hemagglutinating activity with purification was associated with only a slight increase in toxicity.

Hemagglutinin fractions isolated from some other legume species may be more toxic than those from soybeans. Jaffe and Lette (68) found that raw red or black beans (Phaseolus vulgaris) caused a greater reduction in rat growth than several other bean diets tested. The red and black beans were higher in rabbit blood hemagglutinating activity than the other bean varieties, thus implying hemagglutinin as a possible cause of the growth depression. The natal Round Yellow bean (Phaseolus vulgaris) contains

high hemagglutinating activity; however, DEAE-cellulose fractionation showed that this hemagglutinin was not toxic to rats when injected intraperitoneally (138).

Ricin, the caster bean hemagglutinating component, is toxic but the toxicity is probably due to something other than hemagglutinin activity (78, 103, 142). There was a differential loss of hemagglutinating activity and toxicity when ricin was exposed to a variety of reagents (142). Kunitz and McDonald (78) found that the solubility of crystalline ricin resembled the theoretical solubility of a solid solution of two or more components. Further purification employing DEAE-cellulose column chromatography separated ricin into two components (67). The one protein, having a molecular weight of 60,000 was highly toxic to mice. The other protein was the hemagglutinating protein with a molecular weight of 98,000 and possessing little or no toxicity (143).

Saponins

Saponins are bitter-tasting, foam-producing glycosides in which the nonsugar residue (sapogenin) is a triterpenoid alcohol referred to as a soyasapogenol. At least five different saponins have been isolated which exhibit varying degrees of hemolytic and foam-producing activity (152). High levels of soybean saponin also inhibit the proteolytic activities of trypsin and chymotrypsin (66).

Because of their characteristic hemolytic activity and interference with proteolytic activity, it was thought that saponins may be factors contributing to the poor nutritive value of unheated soybean meal (111). However, Birk et al. (12) showed that the hemolytic activity is unaffected by the heat treatment necessary to produce optimum nutritive value of soybean meal, indicating that the hemolytic property of saponins has little or no influence on soybean meal nutritive value. The antiproteolytic activity resulted from a nonspecific reaction of saponins with protein and was readily abolished by the presence of dietary proteins (66).

Pancreatic Enlargement

Growth depression due to feeding raw soybean meal or its fractions is invariably accompanied by an increase in size of the pancreas in chicks (25, 107, 126) and rats (17, 97, 113). This suggests a mechanism whereby the animal compensates for the reduced tryptic activity in the intestine presumably caused by the inhibitors in the raw meal. Chernick et al. (25) found that the pancreases of chicks fed unheated soybean meal rations approximated 1% of their body weights, whereas the organ in those fed heated soybean meal or their regular stock ration never exceeded 0.5%. Rackis (113) showed that the extent of pancreatic hypertrophy in rats depended upon the dietary level of RSBM. At a dietary level of 13% RSBM with 26%

toasted soybean meal as the control, the increment of pancreatic enlargement was 0.07 to 0.14 g/100 g body weight above the control value of 0.79 g/100 g. At a dietary level of 26% raw soybean meal, the pancreas weight increased 0.22 to 0.25 g/100 g body weight above that of the control group. Nesheim et al. (105) claimed that the level of fat in the ration also influenced pancreas size.

This pancreatic enlargement is apparently due to an increased size of the acinar cells with no increase in cell numbers (76). Concentrations of moisture, total lipids, protein, and RNA in the enlarged pancreases of rats were similar to those obtained on heated soybean meal diets, but DNA concentrations were lower. The synthesis of digestive enzymes was higher in chick pancreases adapted to unheated soybean meal diets (124). Histological examinations showed pancreatic hypertrophy to be associated with an accumulation of zymogen granules in the acinar cells (17, 127). An increased secretion of pancreatic enzymes also accompanied pancreatic enlargement (45, 85, 97, 98).

Booth et al. (17) reported that the pancreas was the only organ affected by RSBM diets, but this may not be entirely true. They found no histological changes in the rat heart, liver, thyroid, testes, spleen, kidney, adrenals and intestine. However, a closer examination of several liver and kidney enzymes involved in protein metabolism showed that some functions of these organs were affected

(33). RSBM significantly decreased xanthine dehydrogenase, xanthine oxidase, and arginase activity in chicks and rats as compared to HRSBM. Salman et al. (124) found a higher incidence of intestinal hemmorrhages in chicks consuming unheated soybean meal.

The pancreas rapidly responds to the feeding of raw soybean rations. Chicks showed maximum pancreas enlargement (as percentage of body weight) within 72 hours (126) while rats showed maximum response in 9 days (113). The return to normal size was equally rapid when the raw soybeans in the ration were replaced by heated beans (126).

The pancreas enlargement produced by raw soybeans is apparently associated with its growth depressing effect; however, there is no definite evidence of cause and effect. There is an indication that the pancreas enlargement factor may be associated with soybean hulls (133). When hulls were added to a purified ration, pancreatic enlargement resulted but there was no decrease in weight gains.

Although pancreas enlargement often occurs in rats and chicks fed raw soybeans, evidence indicates that this may not be a general occurrence with all species or all ages. Swine may not develop enlarged pancreases when fed RSBM rations (62). The enzyme activities and relative pancreas size were smaller in shoats fed a RSBM ration than in those fed a commercial soybean ration. Calves fed a 50% soybean protein source milk replacer containing

trypsin inhibitor did not have enlarged pancreases (49). The trypsin and chymotrypsin activities of the pancreases and intestinal contents of these calves were less than those from calves fed other diets. However, it should be noted that this diet caused considerable diarrhea which may have counteracted any raw soybean effect (56). Pancreatic enlargement was less in 5 week-old rats than in those 3 or 4 weeks old (113). Pancreases of older chicks were less affected by RSBM diets than those of younger chicks (107) and adult hens were unaffected by the toxic factor (77) in raw soybeans (128).

Loss of Endogenous Nitrogen

Pancreas enlargement and increased enzyme production by animals fed RSBM suggests that an increased removal of endogenous nitrogen might result (97). Over a period of time, such a loss might increase the animals dietary protein requirements which in turn would account for some of the growth-inhibiting properties of the raw soybeans.

The observation by Lepkovsky et al. (84) that the proteolytic activity in the feces of rats fed RSBM was much higher than in rats fed heated soybean meal supports the above hypothesis. Haines and Lyman (53) and Khazambashi and Lyman (73) observed more protease activity and more TCA-insoluble nitrogen in the intestinal contents of rats fed SBTI or RSBM than in those fed the control diets. The TCA-insoluble protein contained 7 times more essential

amino acids and 17 times more cystine than that of the controls. De Muelenaere (29) observed a similar increase in TCA-insoluble intestinal nitrogen in rats fed SBTI concentrates which was maintained throughout the entire length of the small intestine for 5 hours. He attributed the increase to excessive pancreatic secretions and intestinal mucosal slough-off. However, since growth rates were not measured, no correlations between his findings and affects on growth are possible.

Supplementing diets with methionine, threonine, and valine prevented the growth depression caused by SBTI, but did not prevent the intestinal changes reported above (73). Growth depression due to RSBM was still much more severe than that caused by SBTI when fed at equal trypsin inhibitory levels (53), suggesting that while endogenous nitrogen loss could have contributed to growth depression, other factors in raw soybeans are also involved.

Kwong et al. (80) reasoned that if fecal loss is to explain low nutritive quality, there would have to be either a decreased percentage of nitrogen absorbed as the level of unheated soy flakes in the diet increases or there would have to be a selective failure in the absorption of the limiting amino acid, methionine. They found neither of these situations to exist in rats when unheated soybean flakes were increased from 25 to 75% of the ration.

Metabolic Effects

Evidence indicates that RSBM or its growth depressing fractions influence the conversion of methionine to cystine (3, 79). There is much more cystine in the intestinal contents of these rats than in the contents of rats fed HRSBM (24, 73). Since crystalline trypsin contains 8.7% cystine (4), it follows that the cystine might come from pancreatic trypsin. This high cystine content of trypsin coupled with its enhanced secretion in rats fed RSBM may cause a partial cystine deficiency and thus growth depression.

Some evidence of the influence of RSBM of cystine metabolism is based on amino acid utilization studies. Kwong and Barnes (79) found that feeding raw soybeans increased the expiration of $C^{14}O_2$ by rats given intraperitoneal injections of D-L-methionine-2-C^{14}. The increased metabolism of methionine as measured by CO_2 loss did not occur in rats fed RSBM diets supplemented with cystine. In later experiments using L-methionine-S^{35} they showed that methionine was rapidly converted to cystine in the pancreases of rats given CSBTI via stomach tube (3). The increase in expired CO_2 from methionine by rats on RSBM diets was associated with an increased conversion of methionine to cystine for subsequent incorporation into excretory pancreatic protein.

Borchers (18, 19) reported an increased requirement for threonine and valine in rats fed RSBM that could not be accounted for by decreased protein digestibility or increased loss via fecal loss of pancreatic enzymes. He suggested either decreased intestinal synthesis or increased metabolic requirements as possible explanations of this phenomenon (19).

Impaired Intestinal Digestion and Absorption

Decreased protein digestion and amino acid availability are generally accepted as major factors in explaining the growth inhibition caused by raw soybeans. Supplementing a RSBM diet with methionine or cystine increased the growth rates of rats (58, 59, 79) and chicks (44), but not to the rate of those fed HRSBM. This phenomenon should be explainable by either decreased availability of sulfur amino acids or an increased requirement of RSBM diets. Since methionine is the most limiting amino acid in soy protein for rats (80), chicks (134), and poults (94), both possibilities could create a partial sulfur amino acid deficiency.

Proteins known to be highly undigestible are often improved by steam cooking with some of the principle changes being a loss of cystine, a corresponding appearance of lanthionine, and an increased susceptibility to enzymatic hydrolysis (1). Digestibility studies indicate that

RSBM contains a fraction which becomes digestible only after heating (80, 85, 106). RSBM liberated more sulfide under pressure cooking than HRSBM (1). Fractionation showed that only the water-insoluble residue liberated sulfide while a similar heating of trypsin inhibitors did not. Melnick et al. (102) observed a slower release of methionine in the intestinal tract of rats fed raw versus heated soybeans while the release of lysine was not impaired.

Trypsin inhibitor AA (see Table 3) but not CSBTI decreased intestinal proteolytic activity in chicks (44). This is consistent with previous studies indicating CSBTI is more susceptible to inactivation by acid or pepsin than inhibitor AA (10, 71). Another group (73) obtained increased protease activity in the intestinal contents of rats fed a crude soybean trypsin inhibitor extract.

There is no agreement as to whether the absorption of sulfur amino acids is influenced by heating soybean meal. Early work indicated no difference in the per cent of dietary sulfur which appeared in the feces of rats fed raw or heated soybeans (21, 69). Others claimed just the opposite for both chicks (36) and rats (80). Part of this discrepancy can be accounted for by differences in techniques used. Sometimes (21) digestibility was estimated from the amount of water-insoluble nitrogen in the intestinal tract, which ignores the increased pancreatic secretion associated with unheated soybean meal.

Nesheim and co-workers (22, 42, 105) showed that the absorption of dietary triglycerides and dietary free fatty acids was markedly depressed in chicks fed diets containing unheated soybean protein. This was most pronounced in chicks up to 3 weeks of age but 4-week-old or older chicks showed little impairment. Supplementing the RSBM diet with trypsin (22) or with sodium taurocholate (40) restored fat absorption to normal in 2-week-old chicks. Despite the effectiveness of trypsin supplementation, trypsin inhibitors per se do not appear to be responsible for the poor fat absorption since feeding Kunitz's (77) CSBTI did not result in poor fat absorption (42, 105). The mechanism by which unheated soybean meal depresses fat absorption is unknown. Garlich and Nesheim (42) stated that the decrease is not due to lipase inhibition but sited no data or references. It is not due to a protein or sulfur amino acid deficiency (42).

CHAPTER III

MATERIALS AND METHODS

Experiment I. Effect of Age on Bovine Pancreas Size and Enzyme Activity

Tissue Preparation and Homogenization

Pancreases were obtained from 43 Holstein bull calves varying in age from 1 day to 12 months. They were removed as soon as possible after slaughter, placed on ice, dissected free of connective tissue and large blood vessels, weighed and frozen until assayed for enzyme activity. Pancreases were homogenized in 0.15 M NaCl containing 0.1% Triton-X-100[a] (48). After centrifugation, samples were diluted to contain 0.3 to 1% tissue for activation of trypsinogen and chymotrypsinogen with a crude enteropeptidase preparation[b] at 4°C (48).

Enzyme Assays

Trypsin and chymotrypsin esterase activities were assayed spectrophotometrically with p-toluensulfonyl-L-arginine methyl ester HCl[c] (TAME) and N-benzoyl-L-tryosine

[a]Purchased from Rohm and Hass, Philadelphia, Penn.

[b]Viodenum, produced by Viobin Corp., Monticello, Ill.

[c]Purchased from Cylo Chemical Corp., Los Angeles, Calif.

ethyl ester[c] (BTEE) as substrates, respectively (48). Chymotrypsin assays were performed with 4.7% methanol (v/v) in the reaction mixture.

Statistical Analyses

Animals were grouped by age and the data analyzed for differences among ages using Duncan's multiple rage test (34). Regression analyses were performed on the $\log_{10}$ values of the data to determine the rate of change in pancreas size and its trypsin and chymotrypsin content as functions of body weight (23). The data were fitted in a general regression formula (i.e. $Y=aX^b$) where $Y = \log Y^1$, $X = \log X^1$, a = intercept and b = slope of the regression line. Y^1 is pancreas weight in kg or total pancreatic enzyme content. X^1 is the independent variable, body weight in kg.

Experiment II. Pancreatic Proteolytic Enzymes and Growth of Rats Fed Soybean and Milk Protein Diets

Previous experiments by Gorrill (46) showed that calves grew unsatisfactorily when fed milk replacers containing certain soyflours as part of the protein source. These soyflours contained high levels of soybean trypsin inhibitor, implying that SBTI may be a possible growth inhibitor. Feeding a pure soybean trypsin inhibitor to calves seemed unfeasible and too costly. Thus, experiment II was conducted to see if the rat could be used as a test animal in place of the calf.

Five or six individually housed female weanling rats were fed each of the seven diets listed in Table 4 for 21 days. The ingredients in diets 2, 3, 4 and 7 are given in Table 5. Diet 2 was formulated as a lactose-free diet since diet 1 caused diarrhea in rats. Diets 1, 3, and 5 had been previously fed to calves (46, 49). Diets 1, 3, 4, 5 and 6 were fed simultaneously while diets 2 and 7 were fed at a later date.

At termination the rats were killed by decapitation. Pancreases were removed, weighed, frozen and stored until analyzed for trypsin and chymotrypsin content as described under experiment I.

The small intestine was equally divided into upper and lower sections. Weight of contents from each section and pH of stomach contents were recorded. Intestinal content samples were then frozen and stored until later analysis. A 0.5 to 1 g sample of intestinal contents was used for determining the activities and stabilities of intestinal trypsin and chymotrypsin and in vitro protein digestion. Each sample was diluted to 3 ml with 0.15 N NaCl, then diluted with an equal volume of glycerol and centrifuged at 1300 x g for 30 minutes. One portion of the supernatant was incubated at 37° for 2 hours while the other portion was stored at 4°. Trypsin and chymotrypsin activities were determined in both portions.

TABLE 4.--Dietary treatments.

Diet	Protein	SBTI
	%	units/g
1. All milk replacer[1]	19.2	0
2. Casein replacer	20.5	0
3. Soy protein conc. replacer[2]	20.6	0
4. All milk--ground soybeans[3]	24.2	241
5. High soy replacer[4]	24.2	243
6. All soy replacer[5]	24.2	258
7. High inhibitor replacer[6]	21.3	271

[1]Skim milk and whey powder.

[2]Promosoy, Central Soya, Decatur, Indiana; percentage composition (air dried basis): protein, 71; fat, 0.5; fiber, 3.7; ash, 6.3; and carbohydrate, 18.1.

[3]60% of protein supplied by ground soybeans and 40% of protein supplied by skim milk and whey powder.

[4]60% of protein supplied by a 50% crude protein soybean flour, and 40% of protein supplied by skim milk and whey powder.

[5]100% of protein supplied by a 50% crude protein soybean flour.

[6]100% of protein supplied by Centex, Central Soya, Decatur, Indiana, a 50% crude protein soybean flour known to contain trypsin inhibitor.

TABLE 5.--Composition of diets 2, 3, 4, and 7.

Ingredient	Diet			
	2	3	4	7
	(g)			
Casein	20.5	---	---	---
All milk replacer	---	---	50.8	---
Promosoy[1]	---	25.0	---	---
Centex[1]	---	---	---	43.0
Ground soybeans	---	---	38.2	---
Vitamin-mineral premix[2]	2.5	4.0	1.2	2.5
B vitamin complex[3]	2.0	2.0	0.9	2.0
Trace mineral salt	2.0	2.0	---	2.0
DL-methionine	---	0.5	---	0.5
Aurofac-10[4]	0.25	0.25	0.1	0.25
Corn oil	10.0	---	---	10.0
Fat premix[5]	---	33.0	---	---
Glucose monohydrate[6]	62.75	33.25	8.8	39.75
TOTAL	100.0	100.0	100.0	100.0

[1]Central Soya, Decatur, Indiana.

[2]The premix contained (g); thiamine, 55; menadione, 9.9; vitamin A (30,000 IU/g) + D (2800 IU/g) + E (82 IU/g), 1100; K citrate, 3438; Na_2SeO_3, 0.624; $Al_2(SO_4)_3 \cdot 18H_2O$, 300; H_3BO_3, 10.5; $Na_2MO_4 \cdot 2H_2O$, 10.5; pyridoxine-HCl, 11.6; NaBr, 20.9; ascorbic acid, 57.2; inositol, 286; folic acid, 1.1; p-aminobenzoic acid, 28.6; biotin, 5.5; vitamin B_{12} (.1% trituration of cobalamine), 31.4; (kg) K_2HPO_4, 12.48; Mg), 6.24; and cerelose, 21.3.

[3]Dawes Lab., Inc., Chicago, Illinois, containing (g/lb) riboflavin, 2; pantothenic acid, 4; niacin, 9; and choline chloride, 90.

[4]American Cyanamide Co., Princeton, N. J., containing 10 mg aureomycin 1 lb.

[5]30% fat premix (mixture of dried whey and fat), supplied by milk specialties, Inc., Dundee, Illinois.

[6]Cerelose, Corn Products Company, Argo, Illinois.

In vitro protein digestion was calculated from the change in protein during incubation of intestinal contents for 2 hours at 37°. Nonprotein nitrogen was removed by precipitating protein with 4 volumes of 10% trichloroacetic acid. Precipitates were washed two times with acetone and once with ether before redissolving in 5 volumes of 0.1 N NaOH. Protein was determined spectrophotometrically by methods of Waddell (147) and Tombs et al. (144).

Experiment III. Isolation and Characterization of Growth Inhibitors from Soybeans

Preparation of Soybean Meal Fractions

Raw soybean meal (RSBM) was prepared by grinding soybeans in a Wiley mill using a 2 mm mesh screen and defatting the soybean meal with hexane in a soxhlet apparatus. The solvent was removed by evaporation under a hood at room temperature after which the defatted meal was finely ground in a Wiley mill to facilitate more complete extraction in later steps.

Heated soybean meal (HRSBM) was prepared by autoclaving the raw meal at 101° (4 lbs steam pressure) according to procedures described by Renner and Hill (120). The raw meal was adjusted to about 20% moisture with distilled H_2O and held overnight in a closed container at 4°. It was then spread in layers 1/4 to 3/8 inch thick in metal trays for autoclaving. After autoclaving the meal was air

dried at room temperature, finely ground in a Wiley mill and stored until needed.

The following procedure was used for preparing the various soybean meal fractions. A weighed quantity of RSBM was subjected to the treatment under consideration. Details of these various treatments are given in the results section as they apply to specific trials. The fractions desired for growth inhibition testing were lyophilized to dryness in a Virtis[a] shelf-type lyophilizer, weighed and stored in air tight containers at room temperature until needed for growth assays.

Growth Assay Procedure

The test diet composition is shown in Table 6. The soybean meal source was HRSBM except for the RSBM diet, in which case it was RSBM. The soybean meal fraction being tested replaced part of the HRSBM in the diet. The extent of this replacement was equivalent to the quantity of original RSBM represented by the material being tested. This was determined by the weight of lyophilized sample from a known quantity of starting RSBM. Small adjustments were made for material lost during the isolation procedure. The amounts of each fraction added are shown in Appendix Table II.

Preliminary experiments indicated that weanling mice gave the same type of growth response as weanling rats;

[a]Virtis Research Equipment, New York.

TABLE 6.--Composition of diets.

Ingredient	Amount
	(g)
Salt mix[1]	4.0
Vitamin mix[2]	2.2
Corn oil	5.0
Solka floc[3]	1.5
Cerelose	37.3
Soybean meal source[4,5]	50.0
	100.0

[1]General Biochemicals Corporation, Chagrin Falls, Ohio. Wesson modification of Osborne-Mendel Formula containing (%): $CaCO_3$, 21; $Ca_3(PO_4)_2$, 14.9; $CuSO_4 \cdot 5H_2O$, 0.039; $SePO_4 \cdot 4H_2O$, 1.47; $MgSO_4$, 9.0; $MnSO_4$, 0.02; $K_2Al(SO_4)_2$ $24H_2O$, 0.009; KCl, 12.0; KI, 0.005; KH_2PO_4, 31.0; NaCl, 10.5; NaF, 0.057.

[2]Nutritional Biochemicals Corp., Cleveland, Vitamin diet fortification mixture containing (g/100 lbs diet): vitamin A (200,000 IU/g), 4.5; vitamin D (400,000 IU/g), 0.25; αtocopherol, 5.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride 76.0; menadione, 2.25; p-aminobenzoic acid, 5.0; niacin, 4.5; riboflavin, 1.0; pyridoxine-HCl, 1.0; thiamine-HCl, 1.0; calcium pantothenate, 3.0 (mg/100 lbs. diet): biotin, 20; folic acid, 90; and vitamin B-12, 1.35.

[3]α-Cellulose, Brown Co., Gorham, New Hampshire.

[4]Soybean meal is HRSBM in all cases except for the RSBM diet.

[5]Additions of soybean meal fractions replace part of the HRSBM.

however, the growth response was more rapid and more pronounced. Details of this comparison are given in Appendix Table III. Differences due to dietary treatment were detectable within three days. Thus 21 day-old mice were used in all the growth studies since they required considerably less feed and thus a smaller quantity of fractions prepared from SBM. Males were used except in two trials when females were used. Mice receiving the same diet were housed in the same wire meshed cage. Usually 5 to 7 mice were used per treatment but the number ranged from 3 to 8 depending on the amount of SBM preparation and the resulting feed mixed. Growth studies continued four days or longer if there was sufficient quantities of feed. Beginning and terminating weights as well as daily weights from the third day on were recorded. On the final day mice were killed by decapitation and the pancreas removed, weighed and frozen for later enzyme analyses. Feed intake for each treatment group was estimated by weighing feed initially and after termination of the experiment.

Enzyme Inhibitor Assays

Trypsin and chymotrypsin inhibitor activities of soybean meal and various fractions were determined by measurement of the inhibition of hydrolysis of TAME by trypsin and hydrolysis of BTEE by chymotrypsin, respectively. Inhibitor samples were sufficiently diluted to insure that the assay mixture was not saturated by inhibitor.

The presence of amylase or lipase inhibitors was studied by comparing the effects of whey solutions from HRSBM and RSBM on amylase and lipase activities. Amylase activity was determined by the method of Bernfield (8) wherein the reducing groups liberated from starch are measured by the reduction of 3, 5-dinitrosalicylic acid. Absorbancy was measured at 540 mμ. Lipase activity was determined by the rate of hydrolysis of Tween 20[a] as measured by potentiometric titration. The assay procedure was as follows. To the reaction tube was added 4.0 ml 0.05 M sodium acetate, pH 8.2, 0.5 ml lipase substrate (20 ml 0.2 M sodium acetate, 10 ml Tween 20 and 20 ml H_2O) before adjusting to pH 8.2 with NaOH. Then 0.5 ml enzyme solution (goat lipase plus HRSBM or RSBM extract) was added and the sample titrated to pH 8.2 with .0188 N NaOH using a pH stat to measure the rate of base addition. One unit of activity is equal to 1 micromole of acid produced per minute at 25°.

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[a]Atlas Chemical Industries, Inc., Wilmington, Del.

CHAPTER IV

RESULTS AND DISCUSSION

Experiment I. Effect of Age on Bovine Pancreas Size and Enzyme Activity

Pancreas size, trypsin and chymotrypsin activities for bull calves are summarized in Table 7. All age groups except the one day-old calf had about the same pancreas size and enzyme activity on a body weight basis. The pancreas size and trypsin content of the one day-old group were less than the overall mean for all groups ($P < 0.01$). The most notable difference in the one day-old calf was the higher chymotrypsin activity per mg of pancreas dry matter. Only at this age did the chymotrypsin to trypsin ratio exceed 1.0. This is in agreement with previous studies which indicated that dietary alterations modify chymotrypsinogen synthesis to a greater extent than trypsinogen synthesis (6, 7, 119, 135) and confirmed Gorrill's observation (49) that ruminants generally have a lower chymotrypsin-to-trypsin ratio than most monogastrics.

The regressions of pancreas weight, trypsin and chymotrypsin activities on body weight are illustrated in Figure 1. The regression and correlation coefficients are listed in Table 8. All ages were weighted equally to

TABLE 7.--Pancreas size, trypsin and chymotrypsin activities in Holstein bull calves.

Age	Body wt	Pancreas wt	Trypsin Activity		Chymotrypsin Activity		ChT
			units/mg panc. DM	units/kg body wt	units/mg panc. DM	units/kg body wt	T
	(kg)	(g/kg body wt)					
1 day old (5)[1]	35.2	0.53[c][2]	4.59	596[c]	7.16	920	1.60[a]
2 months (5)	72.3	0.75[abc]	4.95	881[abc]	2.89	506	0.59[b]
4 months (5)	137.9	0.75[abc]	4.28	795[abc]	3.50	623	0.83[b]
5 months (1)	152.4	0.58[c]	4.73	641[bc]	4.17	565	0.88[b]
6 months (3)	183.7	0.91[a]	5.59	1110[ab]	3.96	846	0.75[b]
7 months (5)	198.0	0.81[ab]	4.60	818[abc]	3.89	695	0.85[b]
8 months (6)	220.9	0.70[bc]	5.07	804[abc]	4.08	667	0.83[b]
10 months (5)	275.6	0.81[ab]	6.32	1135[a]	5.96	1107	0.95[b]
11 months (5)	311.2	0.83[ab]	5.25	841[abc]	4.75	698	0.86[b]
12 months (5)	340.5	0.79[ab]	5.86	997[abc]	4.75	340	0.84[b]
Mean		0.75	5.12	870	4.55	759	0.91
$S_{\bar{x}}$[3]		0.05	0.64	143	0.67	499	0.19

[1]Number in parentheses represents the number of animals.

[2]Means in the same column followed by the same letter are not significantly different, $P < 0.05$, using Duncan's multiple range test (34).

[3]Standard error of the mean.

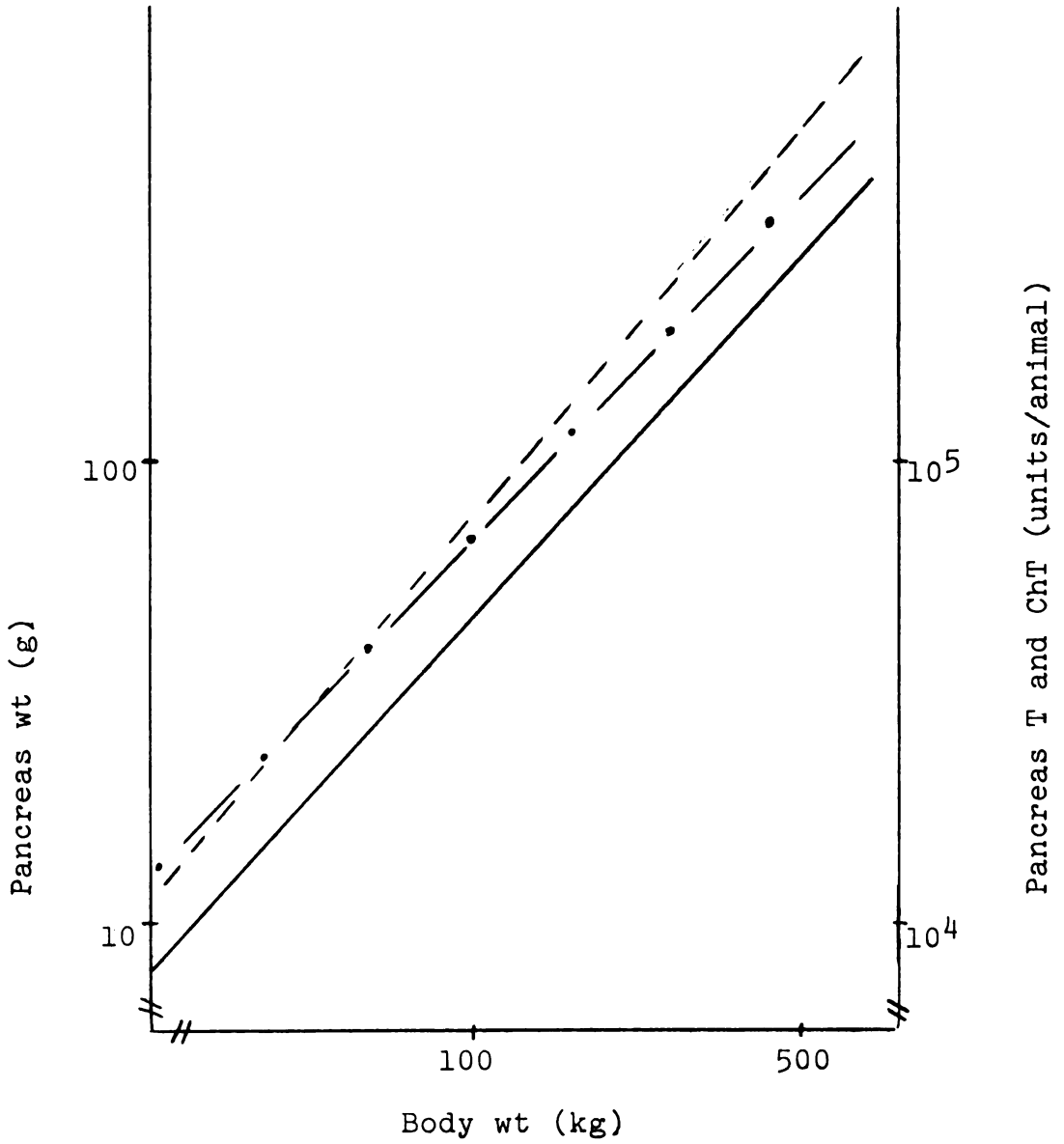

Figure 1. Regressions of pancreas weight, trypsin (T) and chymotrypsin (ChT) on body weight. (———) pancreas weight, (---) trypsin, (—·—) chymotrypsin.

TABLE 8.--Relationships of pancreas weight, trypsin and chymotrypsin to body weight.

Variable	Y intercept a[1]	Regression Coefficient b	Correlation Coefficient r
Pancreas weight			
birth to 12 mo	-3.48	1.16 ± 0.14[2]	0.99
2 to 12 mo.	-3.31	1.08 ± 0.10	0.98
2 to 12 mo.[3]	-3.25	1.06 ± 0.48	0.95
Pancreas trypsin			
birth to 12 mo.	2.52	1.18 ± 0.19	0.98
2 to 12 mo.	2.65	1.13 ± 0.32	0.95
2 to 12 mo.[3]	2.85	1.05 ± 0.50	0.75
Pancreas chymotrypsin			
birth to 12 mo.	2.74	1.06 ± 0.27	0.95
2 to 12 mo.	2.03	1.36 ± 0.30	0.97
2 to 12 mo.[3]	2.25	1.28 ± 0.42	0.83

[1] $Y = aX^b$, where, Y is the dependent variable and X is the independent variable, body weight.

[2] Confidence interval.

[3] Based on actual individual animal values rather than equally weighted age means.

remove any variation due to differences in sample size. Since the newborn calf was the only age group which appeared to possibly deviate significantly from the mean, calculations were made both including and excluding this age group. Calculations were also made using individual, unweighted data on animals two to 12 months of age. The results were not significantly different from the weighted values although variances were greater.

Pancreas weight, trypsin and chymotrypsin content of the pancreas were directly related to body weight as indicated by regression coefficients which were not significantly different from 1.0 ($p > 0.05$). This was consistent with previous calf data (64), but different from the growth responses of several other internal body organs (23). The present data have one exception in that pancreatic chymotrypsin content may increase at a faster rate than body weight from two to 12 months of age. During this age span total pancreas chymotrypsin activity increased somewhat faster than did body weight ($p < 0.2$).

When Gorrill's data (49) on calves from one to six weeks of age were included with these results the following response in pancreatic chymotrypsin activity was observed. At birth the calf pancreas contained high levels of chymotrypsin which rapidly dropped within a week, remained low until about two months of age, then steadily increased, and eventually leveled off to a fairly constant level as

expressed per unit of body weight. The period of lower chymotrypsin activity corresponded to the milk feeding period and thus indicated a dietary adaptation in chymotrypsin synthesis.

Experiment II. Pancreatic Proteolytic Enzymes and Growth of Rats Fed Soybean and Milk Protein Diets

Data on live weight gain, feed intake, size and enzyme content of the pancreas when rats were fed seven different diets are shown in Table 9. Of the four soybean-source diets containing soybean trypsin inhibitor (no. 4-7) only two (no. 5 and 6) reduced weight gains significantly ($P < 0.005$) below that of the no. 3 non-inhibitor soy protein concentrate. Pancreases of rats fed these same four diets (no. 4 to 7) were significantly enlarged ($P < 0.005$), averaging 620 mg/100 g body wt compared to 430 mg/100 g body wt for those from the non-inhibitor diet groups. Pancreatic trypsin and chymotrypsin activity per animal likewise increased ($P < 0.005$) but activity per g wet pancreas tissue remained unchanged by diet. Pancreas size and proteolytic enzyme content were the same on all diets devoid of trypsin inhibitor activity (no. 1 to 3) regardless of protein source, milk or soybean. The unsatisfactory weight gain by rats fed the all milk replacer was apparently due to diarrhea which was probably caused by the high lactose content of the diet. Gains on the casein diet,

TABLE 9.--Body weight changes, feed intakes, size and enzyme content of pancreases of rats fed milk or soybean based diets.

Diet	Body wt change	Feed intake	Pancreas					
			Size	Trypsin Activity		Chymotrypsin Activity		ChT
				Conc.	Total	Conc.	Total	T
	(g/day)	(g/day)	(mg/100g body wt)	(units/g wet wt)	(units/ animal)	(units/g wet wt)	(units/ animal)	
1. All milk	2.02[dl]	13.6[ab]	438[b]	583	290[b]	2079	1095[ab]	3.78
2. Casein	3.18[c]	11.7[b]	460[b]	461	258[b]	1271	699[b]	2.71
3. Soy protein conc.	5.48[a]	14.4[ab]	455[b]	558	442[ab]	1670	1323[ab]	2.99
4. All milk + soybeans	5.02[ab]	14.8[ab]	547[ab]	550	519[ab]	1740	1731[a]	3.34
5. High soy	3.12[c]	13.8[ab]	545[ab]	515	395[ab]	1790	1380[ab]	3.49
6. All soy	4.60[b]	13.8[ab]	657[a]	447	495[ab]	1733	1869[a]	3.78
7. High inhibitor	5.30[a]	15.5[a]	719[a]	679	743[a]	1832	1963[a]	2.70
S_x[3]	0.14	0.7	49	82	100	281	310	0.42
P value[4]	<0.005	<0.005	<0.005	n.s.[2]	<0.05	n.s.	<0.05	n.s.

[1]Means with same superscript are not significantly different at $P < 0.01$ using Duncan's multiple range test (34).

[2]No difference between treatment means, $P < 0.05$.

[3]Standard error of the mean.

[4]Probability of a difference between means arising by chance rather than due to treatment.

which contained cerelose in place of lactose were still unsatisfactory but appeared to be due to a reduced feed intake.

Stomach pH as well as trypsin and chymotrypsin activities in the contents of the small intestine are shown on Table 10. There were differences in pH of stomach contents due to diet but these were apparently not related to protein source or presence of trypsin inhibitor in the soy protein source. Trypsin and chymotrypsin concentrations tended to be higher in the lower than in the upper half of the small intestine but no change due to dietary treatment occurred in one segment that did not occur in the other. Therefore, when the casein and high inhibitor diets were fed, the intestinal contents were not divided into two sections. Apparent total trypsin activity was not significantly affected ($P > 0.10$) by dietary treatments, averaging 137 and 171 units/animal on the SBTI (no. 4 to 7) and non-SBTI diets (no. 1 to 3), respectively. However, chymotrypsin activity was 5 times higher (767 vs 147 units/animal) on the SBTI diets. This indicated that either (a) the SBTI-containing diets inhibited trypsin secretion, or (b) trypsin secretion was increased in the same proportion as chymotrypsin but the enzyme assay procedure used measured only trypsin which was not bound to SBTI. The increase in both trypsin and chymotrypsin in the pancreas was evidence against the first possibility. If the second situation were

TABLE 10.--Stomach pH and trypsin and chymotrypsin activities in intestinal contents of rats fed milk or soybean based diets.

Diet	Stomach pH	Intestinal Contents						
		Trypsin Activity			Chymotrypsin Activity			ChT/T
		Section		Total	Section		Total	
		Upper	Lower		Upper	Lower		
		(units/g contents)		(units/animal)	(units/g contents)		(units/animal)	
1. All milk	3.8[bl]	26.5	57.4	144.8	38.1	71.2	183.8[bl]	1.26
2. Casein	6.0[a]	229.7		194.8	136.4		117.0[b]	0.59
3. Soy protein conc.	4.3[ab]	38.1	87.9	172.0	70.7	43.7	142.5[b]	0.85
4. All milk + soybeans	4.7[ab]	26.1	29.9	141.0	113.6	121.3	592.0[a]	4.20
5. High soy	4.4[ab]	38.8	25.4	108.3	95.5	174.9	636.7[a]	5.88
6. All soy	3.5[b]	40.9	70.2	146.8	200.4	438.9	922.4[a]	6.28
7. High inhibitor	4.9[ab]	61.6		151.0	383.9		918.6[a]	5.91
$S_{\bar{x}}$[3]	0.4			19.0			119.4	
P value[4]	<0.01			n.s.[2]			<0.005	

[1]Means with the same superscript are not significantly different at P < 0.01 using Duncan's multiple range test (34).

[2]No difference between treatment means, P < 0.05.

[3]See footnote 3, Table 9.

[4]See footnote 4, Table 9.

JHE

true, then the rat apparently compensated for the SBTI by secreting a sufficient excess of trypsin so as to maintain a relatively constant level of free trypsin in the intestinal contents.

Protein content of intestinal contents as well as the amount of protein digested in vitro and trypsin and chymotrypsin in vitro stabilities are shown in Table 11. In vitro protein digestion, when expressed on a per g of intestinal content basis, was not affected by dietary treatment. The non-SBTI diets averaged 3.22 mg/g as opposed to 3.14 mg/g for the SBTI diets. The SBTI fed rats tended to have a larger quantity of material remaining in the tract. Therefore, when protein digestion was corrected to a per animal basis, the averages were 6.0 and 10.4 mg protein digested for the total intestinal contents of an animal on the non-SBTI and SBTI diets, respectively. This difference was not significant ($P > 0.10$), but tended to correlate with total proteolytic activity in the intestinal contents (see Table 11) and indicated that intestinal protein digestion was not limiting growth. These results confirmed observations by Scow (131) that the pancreas has a tremendous compensatory capacity and therefore can compensate for SBTI by secreting extra enzymes.

Trypsin stability decreased in intestinal contents of rats fed SBTI diets ($P < 0.005$), averaging 0.88 on

JHE.

TABLE 11.--Protein content and in vitro digestion, and trypsin and chymotrypsin in vitro stabilities.

Interaction		Protein		In vitro enzyme stability[2]	
		Nonincubated conc.	in vitro digestion	Trypsin	Chymo-trypsin
		(mg/g)	(mg/g)		
Diet X intestinal section					
1. All milk	- upper	30.7	3.20	0.90	0.89
	- lower	22.1	1.61	1.01	0.80
	- total	25.4	1.75	0.99a3	0.82bc
	- total (mg/animal)				
2. Casein	- total	22.0	5.86	0.99[a]	0.76[c]
	- total (mg/animal)		6.83		
3. Soy protein conc.	- upper	61.2	11.86	1.05	0.85
	- lower	23.3	-2.14	0.96	0.80
	- total	37.7	2.04	0.98[a]	0.83[bc]
	- total (mg/animal)		4.26		
4. A.M. + soybeans	- upper	38.9	5.64	0.80	0.76
	- lower	22.7	1.01	0.90	0.82
	- total	26.9	2.12	0.88[bc]	0.80[bc]
	- total (mg/animal)		10.53		
5. High soy	- upper	27.9	4.49	0.79	0.92
	- lower	18.9	-1.09	0.80	0.91
	- total	23.0	1.99	0.80[c]	0.91[ab]
	- total (mg/animal)		7.44		
6. All soy	- upper	50.7	11.24	0.91	0.86
	- lower	38.1	-2.14	0.91	0.92
	- total	42.9	2.04	0.92[ab]	0.92[ab]
	- total (mg/animal)		4.26		
7. High inhibitor	- total	30.9	4.33	0.94[ab]	0.98[ab]
	- total (mg/animal)		11.38		
P value[4]		n.s.	n.s.	<0.005	<0.025

[1]Protein digested during a two hour incubation of diluted intestinal contents at 37°.

[2]Ratio of enzyme activity of incubated-to-nonincubated intestinal contents.

[3]Means of total contents in the same column followed by the same superscript are not significantly different at $P < 0.05$ using Duncan's multiple range test (34).

[4]See footnote 4, Table 9.

diets 4 to 7 as opposed to 0.99 on non-SBTI diets (no. 1 to 3). The opposite occurred with chymotrypsin stabilities which averaged 0.90 and 0.80 ($P < 0.005$), respectively. This reflected the fact that SBTI binding reduced the amount of trypsin available for substrate hydrolysis (51, 82). As a result, chymotrypsin was degraded less rapidly especially in the lower half of the small intestine and thus appeared to be more stable on SBTI diets.

Since growth of rats was reduced on only two (no. 5 and 6) of the four (no. 4 to 7) SBTI-containing diets, it appeared as though growth depression was not caused by SBTI. The increased pancreas size and accompanying increased trypsin and chymotrypsin synthesis and secretion associated with all of these SBTI-containing diets indicated a possible compensatory mechanism for combating SBTI effects, but appeared to be unrelated to growth depression. In vitro protein digestion in intestinal contents was unrelated to growth of rats. Thus, impaired protein digestion can also be eliminated from consideration as a mechanism by which soyflour diets 5 and 6 depressed growth.

Experiment III. Isolation and Characterization of Growth Inhibitors from Soybeans

Experiment III was initiated in an attempt to isolate a factor(s) from raw soybean meal (RSBM) which would inhibit growth but be devoid of SBTI activity since the results of Experiment II indicated that SBTI does not

cause the growth depression which results from feeding raw or minimally processed soybean products. This isolation procedure involved numerous extraneous experiments which were all related in some way to the isolation and characterization of growth inhibitory factors in soybeans. These experiments are described in the following pages.

A. Determining the Presence of Digestive Enzyme Inhibitors

The results of trypsin, chymotrypsin, amylase and lipase inhibitor assays on raw soybean meal are shown in Table 12. Each value represents the mean of four determinations. High levels of trypsin and chymotrypsin inhibitors were present as expected. No α-amylase inhibition was detected by comparing the effects of RSBM vs HRSBM extracts on amylase activity. In fact, α-amylase activity was increased in the presence of RSBM extract confirming the presence of previously reported (14) water-soluble amylase. Thus, if an amylase inhibitor is present in soybeans, it cannot be detected by this method. Lipase assays indicated that a trace of lipase inhibitor may be present in soybeans. More stringent assay conditions would be needed to ascertain if this apparent inhibition is real. However, even then it is unlikely that such inhibition would be sufficient to appreciably influence digestion or absorption.

TABLE 12.--Trypsin, chymotrypsin, α-amylase and lipase inhibitor activities in raw soybean meal.

Inhibitor	Activity
	(units/g RSBM)
Trypsin	13,000[1]
Chymotrypsin	2,500[2]
α-Amylase	0[3]
Lipase	4[4]

[1]One unit equals inhibition of hydrolysis of 1 μmole TAME/minute.

[2]One unit equals inhibition of hydrolysis of 1 μmole BTEE/minute.

[3]One unit equals inhibition of liberation of 1 μmole reducing groups/minute.

[4]One unit equals inhibition of 1 μmole acid produced/minute.

B. General Fractionation Procedure

Unheated soybean meal was fractionated as illustrated in Figure 2 to determine whether any of the biological activities could be concentrated in a single component. This procedure is essentially the same as that used by Garlich and Nesheim (42) and Rackis et al. (115), thus providing a check with some previously reported results. Fractionation of unheated soybean meal by this procedure yielded the following distribution of dry matter: water insoluble residue 50.8%, pH 4.4 insoluble proteins 18.8%, pH 8.0 insoluble material 0.8% and total solids in the whey solution (pH 8.0 supernatant) 29.6%. After dialysis and lyophilization, the whey proteins represented 4.9% of the original meal extracted. Two other fractions were also tested in Experiment 1: (a) acetone precipitated whey solution (APWS) and (b) whey solution heated at 60° for 5 minutes (H_{60}WS). The former was prepared as described by Garlich and Nesheim (42) in which two volumes of cold acetone were added to undialyzed whey solution. A small amount of saturated NaCl was added to induce flocculation of the protein. The precipitate was suspended in distilled water and dialyzed against distilled water at 4° for 48 hours. Any insoluble material remaining after dialysis was discarded before lyophilizing the dialyzed solution. The H_{60}WS fraction was prepared by heating

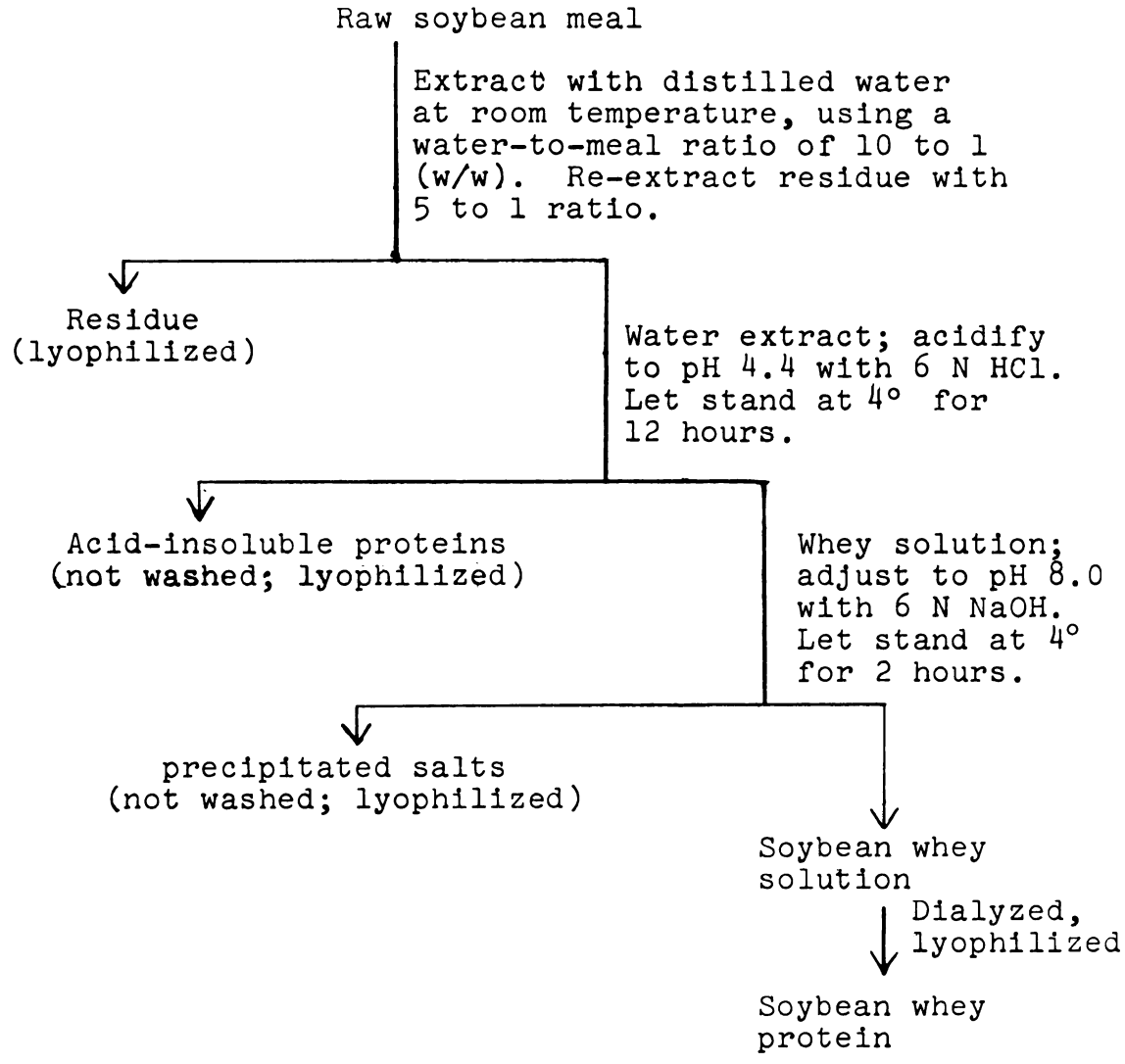

Figure 2. Fractionation procedure of raw soybean meal.

undialyzed whey solution in 200 ml volumes to 60° for five minutes then rapidly cooling in an ice bath.

The results of Experiment 1 are shown in Table 13. The loss of weight by mice fed the water-insoluble residue indicated that (1) the growth inhibitor was water insoluble or (2) the extraction was incomplete. Other investigators have noted reduced weight gains and feed efficiencies when the water insoluble residue was fed to rats (117) or chicks (42); however, the reductions were not as marked as shown here. In this experiment the RSBM was not finely ground after hexane extraction and apparently incomplete extraction was the most probable cause of the marked growth reduction by this group especially since the growth inhibitor(s) may not be readily soluble. The RSBM was finely ground before use in all subsequent experiments. Growth rates were reduced slightly in all the other fractions tested, with the weight reduction being most pronounced in mice fed derivatives of the pH 8.0 supernatant (diets 3, 7 and 8). Heating the whey solution 5 minutes at 60° did not remove or destroy the growth inhibitor activity but did destroy considerable amounts of SBTI. Much of the growth reduction on the RSBM and residue diets was due to low feed intake as indicated by the marked reduction in per cent growth inhibition when corrected for feed intake. However, no group consumed as much feed as the positive control HRSBM. Mice fed diets containing higher levels of

TABLE 13.--Response of mice when fed HRSBM, RSBM, or several fractions of RSBM.

Dietary Treatment[1]	Wt gain	Pancreas wt		Feed Efficiency[2]	SBTI	Growth Inhibition	
	g/day	mg	% of body wt		units/g diet	%[3]	% Corrected for intake[4]
1. HRSBM (7)[5]	0.90[a6]	112[b]	0.63[c]	0.20	0	---	---
2. RSBM (6)	-0.49[bc]	74[c]	0.76[bc]	-0.30[c]	6500	154	65
3. Dial. whey solution (3)	0.52[a]	130[ab]	0.99[ab]	0.14[ab]	915	42	34
4. Residue (3)	-0.20[bc]	114[ab]	1.19[a]	-0.07[b]	140	122	74
5. pH 4.4 ppt. (5)	0.73[a]	125[ab]	0.77[bc]	0.17[ab]	37	19	18
6. pH 8.0 ppt. (3)	0.83[a]	97[bc]	0.62[c]	0.21[a]	2	8	7
7. Acetone ppt. W.S. (3)	0.67[a]	148[a]	1.02[ab]	0.16[ab]	605	26	24
8. H_{60} Whey solution (6)	0.26[b]	115[ab]	0.85[bc]	0.11[ab]	192	71	56
$S_{\bar{x}}$[7]	0.15	10	0.08	0.07			

[1]Each diet fed six days.

[2]Weight gain divided by feed intake

[3]Per cent growth inhibition = (wt gain on HRSBM diet - wt gain on test diet/wt gain on HRSBM diet) x 100.

[4]Per cent growth inhibition x feed intake on test diet/feed intake on HRSBM diet.

[5]Number in parenthesis represents number of animals.

[6]Figures followed by the same letter in that column are not significantly different, $P < 0.05$, using Duncan's multiple range test (34).

[7]Standard error of the mean.

SBTI tended to have enlarged pancreases, except for the RSBM diet group. Starvation was probably an overriding factor on that diet. Since only solubilized SBTI can be measured by the inhibitor assay used, SBTI values for water-insoluble residue diets are always low.

Experiment 1 was repeated using finely ground RSBM which enabled more efficient water extraction. The results (Experiment 2) are shown in Table 14. Extraction of RSBM was more complete; however, almost half of the growth inhibitor activity remained in the water-insoluble residue. In the next fractionation step most of the growth inhibitor activity remained in the pH 4.4 supernatant. However, if SBTI activity can serve as an indication of "cleanness" of separation between precipitate and supernatant, growth inhibitor activity was not completely separated at this step. Similarly, in the next step (pH 8.0 precipitation) growth inhibitor activity was equally divided between the two fractions while SBTI was concentrated in the supernatant. This experiment indicated that growth inhibitor activity present in the pH 8.0 supernatant was not dialyzable; however, experiments reported later cast some doubt on that observation.

C. Water-Insoluble Residue Studies

Almost half of the growth inhibitor activity remained in the water-insoluble fraction indicating that water extraction may not be the most effective method for removing

TABLE 14.--Response of mice when fed HRSBM, RSBM, or several fractions of RSBM in Experiment 2.

Dietary Treatment[1]	Wt Gain	Pancreas wt		Feed Efficiency[2]	SBTI	Growth Inhibition	
	g/day	mg	% of body wt		units/g diet	%[3]	% corrected for intake[4]
Experiment a							
1. HRSBM (5)[5]	0.92[a6]	86	0.59	0.25[a]	0	---	--
2. RSBM (5)	-0.39[c]	75	0.72	-0.23[c]	6500	142	64
48. Residue (5)	0.44[b]	88	0.64	0.13[b]	170	52	49
5. pH 4.4 ppt (5)	0.75[ab]	86	0.60	0.19[ab]	40	18	19
6. pH 8.0 ppt (5)	0.65[ab]	88	0.62	0.16[ab]	2	29	31
$S_{\bar{x}}$[7]	0.10	5	0.04	0.03			
Experiment b							
1. HRSBM (7)	0.58[a]	68	0.51[c]	0.24	0	---	--
60. pH 4.4 super (7)	0.26[b]	70	0.63[ab]	0.13	3650	55	44
64. pH 8.0 super (7)	0.40[ab]	66	0.54[bc]	0.16	3500	31	31
65. Dial. whey soln (7)	0.40[ab]	86	0.70[a]	0.16	3340	31	31
$S_{\bar{x}}$[7]	0.10	5	0.03	0.04			

[1]Each diet fed four days.

[2-7]See footnotes 2-7, respectively, Table 13.

these factors from RSBM. Therefore, studies were conducted to (a) determine the solubility of the water-insoluble residue in various organic and inorganic solvents, (b) determine the effects of other extraction procedures and/or digestions on the growth inhibitor activity in the water-insoluble residue and (c) determine the effects of other solvents on the efficiency of growth inhibitor extraction from RSBM.

1. Solubility studies.--Twenty ml of each of the solvents listed in Table 15 were added to 1 g of water-insoluble residue in a 50 ml Erlenmeyer flask. Samples were shaken one hour on a wrist-action shaker.[a] They were observed for visible solubility, protein in the supernatant and SBTI activity in some of the supernatants.

The water-insoluble residue was essentially insoluble in all the solvents tested with the possible exception of pepsin and papain solutions which yielded slightly cloudy-white supernatants. Protein and SBTI determinations indicated that only a small amount of protein was dissolved by some of the solvents but that dissolved was high in SBTI activity. Even the most efficient solvents dissolved no more than 20% of the protein present in the residue. There was no difference in SBTI activity of the solutions assayed with the possible exception of improved extraction efficiency using 0.15 N NaCl.

[a]Burrell Co., Pittsburgh, Penn.

TABLE 15.--Solubility of the water-insoluble residue in various solvents.

Solvent	Protein	SBTI
	(mg/ml solvent)	(units/g residue)
Water	1.2	395
Acid, pH 2.0	1.2	---
Acid, pH 5.0	1.2	---
Base, pH 9.0	1.4	242
Detergent, 1% Triton-X-100	---	---
1% Triton-X-100 in 0.15 N NaCl	5.2	---
0.15 N NaCl	3.8	563
Pepsin in 0.01 N HCl	1.8	310
Papain in H_2O	4.8	382
Acetone	---[1]	---[1]
Benzene	---[1]	---[1]
Carbon tetrachloride	---[1]	---[1]
Methanol	---[1]	---[1]
Pentane	---[1]	---[1]
Toluene	---[1]	---[1]

[1]Unable to determine because of solvent interference with assay procedure.

2. Digestion and extraction effects on growth inhibition.--Several of the extraction solvents which showed some promise in the solubility studies were used with larger quantities for growth inhibitor activity studies. Forty-five g samples of water-insoluble residue were extracted with 600 ml of water, 0.15 N NaCl, pepsin (4.5 g/600 ml) at pH 2 or papain (4.5/600 ml) for two hours. All extractions were conducted at 25° with the exception of pepsin digestion which was conducted at 37°. Samples were centrifuged followed by lyophilization of the separated precipitate and supernatant fractions.

Results when these preparations were fed to mice are given in Table 16. The original water-insoluble residue caused decreased growth rates ($P < 0.10$) with only a slight decrease in intake and decreased feed efficiency ($P > 0.10$) compared to the HRSBM diet. Extracting the residue with water, 0.15 N NaCl, or digestion with pepsin or papain did not remove or destroy the factor(s) responsible for reduced growth and reduced feed efficiency. Growth rates of mice fed re-extracted residue fractions were less ($P < 0.005$) than those of mice fed extract fractions. Pancreas size was not affected by any of the treatments.

3. Raw soybean meal extraction studies.--In conjunction with extraction experiments on the water-insoluble residue, similar extractions of RSBM were also conducted to determine if water is actually the most efficient solvent

TABLE 16.--Response of mice when fed extraction or digestion preparations of the water-insoluble residue from raw soybean meal.

Dietary Treatment[1]	Wt. gain	Pancreas wt		Feed Efficiency[2]	SBTI	Growth Inhibition	
	g/day	mg	% of body wt		units/g diet	%[3]	% corrected for intake[4]
1. HRSBM (5)[5]	0.81[a6]	117	0.72	0.25[a]	0	---	---
2. RSBM (5)	-0.15[c]	65	0.63	-0.06[d]	6500	119	96
21. Residue (5)	0.47[ab]	82	0.61	0.18[abc]	260	42	34
22. Res.,[9] H_2 super (5)	0.81[a]	91	0.62	0.25[a]	170	0	0
23. Res., H_2O ppt (5)	0.52[ab]	90	0.66	0.17[abc]	---[8]	36	35
24. Res., NaCl super (5)	0.81[a]	98	0.63	0.25[a]	260	0	0
25. Res., NaCl ppt (5)	0.45[ab]	91	0.63	0.16[ab]	---[8]	44	38
26. Res., pepsin super (5)	0.89[a]	76	0.58	0.26[a]	135	0	0
27. Res., pepsin ppt (5)	0.52[ab]	89	0.61	0.20[ab]	---[8]	36	29
28. Res., papain super (5)	0.80[a]	92	0.63	0.25[a]	240	1	1
29. Res., papain ppt (5)	0.23[b]	86	0.61	0.10[abc]	---[8]	72	54
$S_{\bar{x}}$[7]	0.13	9	0.05	0.02			

[1]Each diet fed six days.

[2-7]See footnotes 2-7, respectively, Table 13.

[8]Could not assay since sample was insoluble.

[9]Res. equals water-insoluble residue.

for extracting the factors under study. Fifty g of RSBM were extracted for three hours with water, 0.15 N NaCl, 1% Triton-X-100, or chloroform-methanol (2/1, v/v) at pH 2, pH 7 and pH 10. The chloroform-methanol ($CHCl_3$-MeOH) extractions at pH 2 and 10 were achieved by adjusting the pH of a water-RSBM slurry than adding the $CHCl_3$-MeOH. Dry RSBM was used with $CHCl_3$-MeOH for the neutral extraction procedure. The purpose of the $CHCl_3$-MeOH extractions was an attempt to extract the compound(s) responsible for the reduced feed intake on RSBM diets. After the extraction period all samples were centrifuged, the precipitates washed once with solvent and lyophilized.

Data in Table 17 illustrate the efficiency of growth inhibitor extraction by these solvents. Water was slightly more efficient ($P > 0.10$) than 0.15 N NaCl and 1% Triton-X-100 in extracting the growth inhibitor from RSBM. Chloroform-methanol extraction did not remove the factor(s) causing reduced feed consumption. In fact, intake was markedly reduced on all $CHCl_3$-MeOH extracted RSBM diets, particularly diets 37 and 38. Intake was somewhat higher on diet 39 (1.3 g/day vs 3.2 g/day per animal on diet 1) so the pH 10 $CHCl_3$-MeOH extraction was repeated (diet 41). Intake was still low and the extraction efficiency of growth inhibitor activity still below that of water alone.

TABLE 17.--Efficiency of growth inhibitor extraction from RSBM by several solvents.

Dietary Treatment[1]	Wt. gain	Pancreas wt		Feed Efficiency[2]	SBTI	Growth Inhibition	
	g/day	mg	% of body wt		units/g diet	%[3]	% corrected for intake[4]
1. HRSBM (5)[5]	0.79[a6]	84	0.59	0.30[a]	0	---	---
2. RSBM (5)	-0.27[c]	70	0.76	-0.17[bc]	6500	134	84
21. H_2O extracted RSBM (2)	0.51[ab]	91	0.78	0.17[a]	---[8]	35	41
43. NaCl extracted RSBM (5)	0.27[b]	66	0.58	0.12[ab]	---[8]	66	56
44. Triton-X-100 ext'd RSBM (5)	0.33[b]	77	0.65	0.14[a]	---[8]	58	63
$CHCl_3$ MeOH extracted RSBM at:							
38. pH 2 (5)	-0.51[c]	68	0.70	-0.15[bc]	---[8]	165	17
37. pH 7 (5)	-1.01[d]	56	0.69	-0.34[c]	---[8]	228	20
39. pH 10 (5)	-0.65[c]	51	0.54	-0.05[bc]	---[8]	121	50
41. pH 10 (5)	-0.31[c]	65	0.73	-0.31[c]	---[8]	139	53
$S_{\bar{x}}$[7]	0.09	4	0.07	0.08			

[1]Diets fed four days.

[2-7]See footnotes 2-7, respectively, Table 13.

[8]Could not assay since sample was insoluble.

D. Gastrointestinal Enzyme Digestions

Raw soybean meal samples were digested with pepsin, amylase, lipase or trypsin to determine if normal gastrointestinal enzyme action would destroy growth inhibitor activity. Fifty g of RSBM were digested in 600 ml of enzyme solution for four hours at 35-37°. Samples were then centrifuged, the precipitate washed once with distilled water, the washings added to the supernatant fraction and the precipitate and supernatant fractions lyophilized. Pepsin digestion was conducted using 0.6 g pepsin[a] at pH 2.0. Amylase digestion was accomplished using 0.5 g of amylase.[b] Lipase digestion was conducted using 0.5 g of crude hog pancreas lipase[c] in 0.05 M $CaCl_2$ solution at pH 8.0. Trypsin digestion was carried out using 0.35 g of crystalline trypsin[d] and 2.5 g of crude trypsin[e] in 0.05 M $CaCl_2$ solution at pH 8.0. Sufficient amounts of trypsin were added to tie up essentially all the SBTI present in the RSBM and yet provide an excess of trypsin for digestion.

---

[a]Merck and Co., Rahway, N.J.

[b]Mylase S.A., a fungal amylase, Wallenstein Co., Inc., New York.

[c]Sigma Chemical Co., St. Louis, Mo.

[d]Trypsin, bovine pancreas, 2x crystalline, Nutritional Biochemicals Corp., Cleveland, Ohio.

[e]Fairchild Bros. and Foster, New York.

The results (Table 18) when the products from enzyme digestions were fed to mice indicated that growth inhibitor activity was not destroyed by the digestive enzyme treatments tested. Growth rates of mice fed precipitate or supernatant digestion fractions were less ($P < 0.005$) than of those fed HRSBM. Amylase and trypsin supernatant fractions reduced growth rates more ($P < 0.005$) than the respective precipitate fractions. The supernatant from pepsin digestion contained very little growth inhibitory activity which might be interpreted to mean destruction; however, the total amount of growth inhibitor activity (per cent of growth inhibition corrected for feed intake) in RSBM could be accounted for by summation of that in the pepsin digested precipitate and supernatant. SBTI was concentrated in the supernatant fractions as expected from previous experiments. Pancreases of mice fed these supernatant diets were larger (expressed as per cent of body weight) than those of mice fed HRSBM indicating that SBTI may be affecting pancreas size. However, the trypsin digested supernatant fraction contained considerable growth inhibitor activity and caused enlarged pancreases (expressed as per cent of body weight) despite the fact that its SBTI was in a bound form. Feed efficiencies were reduced on all RSBM treatment diets, but the reductions were most pronounced on those containing enzyme-digested supernatant fractions and RSBM.

TABLE 18.--Response of mice when fed gastrointestinal enzyme digested fractions of raw soybean meal.

Dietary Treatment[1]	Wt gain	Pancreas wt		Feed Efficiency[2]	SBTI	Growth Inhibition	
	g/day	mg	% of body wt		units/g diet	%[3]	% corrected for intake[4]
1. HRSBM (5)[5]	0.92[a6]	86	0.59[f]	0.25[a]	0	---	---
1. HRSBM (5)[8]	0.89[ab]	190	0.71[cdef]	0.16[abc]	0	---	---
2. RSBM (5)	-0.39[g]	75	0.72[cdef]	-0.23[h]	6500	142	64
2. RSBM (5)[8]	-0.32[fg]	173	0.86[ab]	-0.10[g]	6500	136	55
46. Pepsin digested ppt (5)	0.56[bc]	88	0.63[ef]	0.18	199	39	32
58. Pepsin digested super (5)	0.49[cd]	191	0.84[abc]	0.09[cd]	1230	45	33
45. Amylase dig ppt (5)	0.58[abc]	91	0.64[ef]	0.20[ab]	120	37	29
57. Amylase dig super (5)[8]	0.01[ef]	176	0.79[abcd]	-0.00[ef]	1250	101	79
42. Lipase dig ppt (5)	0.29[cde]	86	0.69[def]	0.09[cd]	430	68	58
49. Lipase dig super (5)	0.08[e]	87	0.76[bcde]	0.02[def]	1230	91	80
47. Trypsin dig ppt (5)	0.17[de]	81	0.66[def]	0.05[de]	90	82	72
59. Trypsin dig super (5)[8]	-0.41[g]	194	0.92[a]	-0.08[fg]	110	146	93
$S_{\bar{x}}$[7]	0.10	5	0.04	0.04			

[1]Each diet fed four days, three days for groups with superscript 8.

[2-7]See footnotes 2-7, respectively, Table 13.

[8]Treatments with this superscript were tested at a different time than the other treatments and are likewise compared among themselves statistically.

E. Reasons for Reduced Consumption of RSBM

Feed consumption was always less on RSBM diets than on any other diets tested. Intake of some RSBM fractions tested was often slightly less than that of the positive control group (i.e., HRSBM); however, never as low as that of the RSBM fed group. Thus, it was postulated that RSBM may contain some compound, either volatile or readily absorbed, which "tells" the animal not to eat the feed, and that this compound was rather easily destroyed or inactivated by any of the fractionation procedures previously used. A volatile compound should be removed by heating a dry sample under vacuum. Also, compounds carrying negative charges such as fatty acids should be extracted by a solvent such as $CHCl_3$-MeOH at pH 2 while positively charged compounds such as amines should be extractable at pH 10.

To test this theory a limited study was conducted in an attempt to remove the cause of reduced feed consumption from the RSBM. The vacuum heated RSBM was prepared by heating 75 g of dry RSBM at 110° for 18 hours under vacuum in the lyophilizer. The $CHCl_3$-MeOH extracted diet indicated that the intake depressant may have been removed. Consequently, this treatment was repeated in experiment c and the results were just the opposite. The very low consumption of diets 37 and 38 may be partially attributed

TABLE 19.--Effects of several treatments on feed consumption

Dietary Treatment	Feed Intake (g/day/animal)
Experiment a[1]	
1. HRSBM (5)[2]	3.2
2. RSBM (5)	2.6
36. Vacuum heated RSBM (4)	1.2
Experiment b[1]	
2. RSBM (3)	1.0
37. $CHCl_3$-MeOH extracted dry RSBM (5)	0.3
38. pH 2.0 $CHCl_3$-MeOH extracted RSBM (5)	0.3
39. pH 10.0 $CHCl_3$-MeOH extracted RSBM (5)	1.3
Experiment c[1]	
1. HRSBM (5)	2.6
2. RSBM (5)	1.6
41. pH 10.0 $CHCl_3$-MeOH extracted RSBM (5)	1.0

[1]Experiments a, b and c continued for 5, 3 and 4 days, respectively.

[2]Number in parentheses represents number of animals.

to the fact that the extracted RSBM was still moist with a slight chloroform odor when the experiment was started.

F. Fractionation of Acetone Precipitated Whey Solution

Acetone precipitated whey solutions were prepared a as previously described in section B. In experiment 3, 50% and 100% $(NH_4)_2SO_4$ saturated preparations were prepared by adding 228 g and 456 g of $(NH_4)_2SO_4$, respectively, to 734 ml of acetone precipitated whey solution (APWS). The precipitates were redissolved in distilled water then the precipitate and supernatant fractions were dialyzed against distilled water. The bentonite-celite supernatant fraction was prepared as described by Honovar et al. (61). These authors stated that this procedure selectively adsorbed trypsin inhibitors on the bentonite-celite while hemagglutinins remained in the supernatant. Eight and eight-tenths g of bentonite-celite (1:1, w/w) were added to 734 ml of APWS, the mixture was stirred overnight at 4°, centrifuged and the supernatant lyophilized. The heated fractions were prepared by heating 734 ml of APWS in 15 ml aliquots in tall test tubes for 1, 5 and 10 minutes in a boiling water bath and then cooling immediately in an ice bath.

The results of Experiment 3 are shown in Table 20. While growth rates of mice fed APWS were significantly less ($P < 0.05$) than of those fed HRSBM, a substantial

TABLE 20.--Response of mice when fed bentonite-celite, $(NH_4)_2SO_4$, or heat treated preparations of acetone precipitated whey solution.

Dietary Treatment[1]	Wt. gain	Pancreas wt		Feed Efficiency[2]	SBTI	Growth Inhibition	
	g/day	mg	% of body wt		units/g diet	%[3]	% corrected for intake[4]
1. HRSBM (5)[5]	1.16[a6]	80[c]	0.49[d]	0.33[a]	0	---	---
12. APWS (5)	0.75[bcd]	108[b]	0.85[ab]	0.24[abc]	1150	36	31
13. 100% $(NH_4)_2SO_4$ super (5)	0.99[ab]	96[bc]	0.66[c]	0.28[abc]	0	15	15
14. 100% $(NH_4)_2SO_4$ ppt (5)	0.53[d]	133[ab]	0.92[a]	0.18[bc]	1650	54	46
15. 50% $(NH_4)_2SO_4$ super (5)	1.09[ab]	100[bc]	0.65[cd]	0.31[ab]	460	6	6
17. Bentonite-celite (5) supernatant	0.85[abcd]	94[bc]	0.68[c]	0.24[abc]	630	27	27
18. 1 min. Heated super (5)	0.51[d]	96[bc]	0.69[bc]	0.16[c]	640	56	52
19. 5 min. Heated super (5)	0.84[abcd]	99[bc]	0.74[bc]	0.24[abc]	670	28	28
20. 10 min. Heated super (5)	0.74[bcd]	111[ab]	0.81[abc]	0.21[abc]	570	36	36
$S_{\bar{x}}$[7]	0.13	7	0.05	0.04			

[1]Each diet fed for four days.

[2-7]See footnotes 2-7, respectively, Table 13.

amount of the growth inhibitor activity was apparently lost or destroyed prior to this step. Of the growth inhibitor activity present in the APWS, most of it appeared to be precipitated by 50% $(NH_4)_2SO_4$ saturation, but this sample was lost and therefore not tested. Verification of these results had to await completion of the next experiment. Although the five minute heated sample gave inconclusive results, the one and ten minute heated samples indicated that the growth inhibitor, when in solution at approximately neutral pH, was not destroyed by heating, but one-half of the SBTI activity was destroyed. The bentonite-celite treatment results were inconclusive, but there was no evidence to indicate preferential adsorption of trypsin inhibitor over growth inhibitor. Pancreas enlargement appeared to be directly related to dietary SBTI activity since removal or denaturation by bentonite-celite adsorption or by heat treatments resulted in a concomitant reduction in pancreas size relative to body weight.

Experiments 4 and 5 were conducted to further clarify the effects of the treatments tested in Experiment 3. Experiment 4 repeated the 50% $(NH_4)_2SO_4$ treatments and the bentonite-celite treatment with an additional step. Five hundred ml of APWS was stirred with 8.0 g of bentonite-celite (1:1, w/w) overnight at 4°, centrifuged and the precipitate discarded. The supernatant was brought to 75%

$(NH_4)_2SO_4$ saturation by adding 232 g $(NH_4)_2SO_4$ to 450 ml of solution. After centrifugation, the precipitate was dissolved in distilled water. Precipitate and supernatant solutions were both dialyzed against distilled water and lyophilized. In Experiment 5 APWS was divided into three fractions. 25% and 80% $(NH_4)_2SO_4$ precipitates, and 80% $(NH_4)_2SO_4$ supernatant. The intent was to concentrate SBTI in the 80% $(NH_4)_2SO_4$ precipitate.

The results of Experiments 4 and 5 are shown in Table 21. Weight gains in Experiment 4 were based on the first four days growth even though the experiment continued for six days. This was because some groups were short of feed the sixth day which interferred with the dietary effects on weight gains. The growth inhibitor and SBTI remaining in the supernatant of the bentonite-celite treated APWS were concentrated in the 75% $(NH_4)_2SO_4$ saturation precipitate (diet 33, Table 21) which partially confirmed the results of Honovar et al. (127). However, considerable growth inhibitor activity and SBTI were either destroyed or retained on the bentonite-celite. The 50% $(NH_4)_2SO_4$ saturation treatment did not completely separate growth inhibitor activity or SBTI into one fraction but tended to concentrate both, particularly SBTI, in the $(NH_4)_2SO_4$ precipitate. Experiment 5 indicated that most SBTI precipitated between 25% and 80% $(NH_4)_2SO_4$ saturation while the factor(s) responsible for most of the growth

TABLE 21.--Response of mice when fed bentonite-celite and $(NH_4)_2SO_4$ treated preparations of acetone precipitated whey solution.

Dietary Treatment[1]	Wt. gain	Pancreas wt.		Feed Efficiency[2]	SBTI	Growth Inhibition	
	g/day	mg	% of body wt		units/g diet	%[3]	% corrected for intake[4]
Experiment 4							
1. HRSBM (5)[5]	0.98^{a}[6]	117^{ab}	0.72^{abc}	0.21	0	---	---
35. APWS (4)	0.51^{c}	107^{ab}	0.79^{ab}	0.16	860	48	40
30. 50% $(NH_4)_2SO_4$ super (4)	0.76^{b}	101^{b}	0.67^{bc}	0.17	230	22	23
31. 50% $(NH_4)_2SO_4$ ppt (4)	0.69^{b}	113^{ab}	0.82^{ab}	0.13	430	30	31
32. Bent.-cel. $(NH_4)_2SO_4$ super (4)	1.06^{a}	92^{b}	0.60^{c}	0.15	5	0	0
33. Bent.-cel. $(NH_4)_2SO_4$ ppt (4)	0.75^{b}	132^{a}	0.85^{a}	0.20	490	24	23
$S_{\bar{x}}$[7]	0.02	9	0.05	0.02			
Experiment 5							
1. HRSBM (7)	0.72^{ab}[6]	111	0.75	0.20^{a}	0	---	---
51. 25% $(NH_4)_2SO_4$ ppt (7)	0.85^{a}	128	0.80	0.24^{a}	50	0	0
52. 80% $(NH_4)_2SO_4$ ppt (8)	0.61^{b}	119	0.77	0.17^{ab}	320	15	15
56. 80% $(NH_4)_2SO_4$ super (7)	0.38^{c}	98	0.71	0.11^{b}	160	47	47
$S_{\bar{x}}$[7]	0.06	9	0.05	0.02			

[1]Each diet fed for six days. Weight gains are for first four days in Experiment 4.

[2-7]See footnotes 2-7, respectively, Table 13.

inhibition remained in the 80% $(NH_4)_2SO_4$ supernatant. Pancreases of mice fed the higher SBTI containing diets tended to be larger (as per cent of body weight) but the trend was not as definite as in some other experiments.

Growth rates of mice on the 80% $(NH_4)_2SO_4$ precipitate and supernatant diets indicated that a partial separation of growth inhibitor activity from SBTI had been achieved. However, these results were inconsistent with the 50% $(NH_4)_2SO_4$ and bentonite-celite-$(NH_4)_2SO_4$ results. This prompted the conclusion that the differential $(NH_4)_2SO_4$ precipitation techniques used did not separate the biological activities sufficiently for satisfactory progress.

G. Growth Inhibition Due to Crystalline Soybean Trypsin Inhibitor

Crystalline soybean trypsin inhibitor[a] was added to diets for mice at three levels to ascertain its affects on growth rates. This study was conducted simultaneously with Experiment 5 (see Table 21).

The results of this experiment are shown in Table 22. Growth rates were reduced ($P < 0.05$) only by the two highest levels of SBTI in experiment a, but were not reduced ($P > 0.05$) by the highest SBTI level in experiment b. The growth inhibitory effect of crystalline SBTI was

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[a]Soybean trypsin inhibitor, five times crystallized, Nutritional Biochemicals Corp., Cleveland, Ohio.

TABLE 22.--Response of mice to feeding three different levels of crystallized soybean trypsin inhibitor.

Dietary Treatment[1]	Wt. Gain	Pancreas wt		Feed Efficiency[2]	SBTI	Growth Inhibition	
	g/day	mg	% of body wt		units/g diet	%[3]	% corrected for intake[4]
Experiment a							
1. HRSBM (7)[5]	0.72[a6]	111	0.75	0.20[a]	0	--	--
52. 80% $(NH_4)_2SO_4$ppt (8)	0.61[ab]	119	0.77	0.17[ab]	140	15	15
53. 80% $(NH_4)_2SO_4$ppt + SBTI(8)	0.40[b]	104	0.76	0.11[b]	280	44	44
54. SBTI (8)	0.54[ab]	100	0.73	0.15[ab]	140	25	25
55. 4 x SBTI (7)	0.42[b]	114	0.83	0.12[b]	550	42	42
$S_{\bar{x}}$[7]	0.06	9	0.05	0.02	--	--	--
Experiment b							
1. HRSBM (8)	0.63	89	0.63	0.21	0	--	--
55. 4 x SBTI (7)	0.51	101	0.72	0.16	550	19	20
$S_{\bar{x}}$[7]	0.06	6	0.04	0.02			

[1]Each diet fed six and five days in experiments a and b, respectively.

[2-7]See footnotes 2-7, respectively, Table 13.

somewhat additive since adding an equal number of SBTI units to diet 52 more than doubled growth inhibitor activity (diet 53). However, increasing the SBTI activity to four times the level present in diets 52 and 54 did not quadruple growth inhibition. The pancreas was not enlarged ($P > 0.05$) by any of the SBTI-containing diets. Diet 55 was repeated at a later date (experiment b). Growth rates and feed efficiencies were reduced slightly ($P > 0.05$) and pancreases were enlarged slightly ($P > 0.05$). Thus it appears that SBTI may cause some growth depression, but the amount of depression is not nearly as great as that caused by RSBM.

H. Acetic Acid Extraction of RSBM

In previous experiments there was some difficulty in lyophilizing some of the undialyzed fractions to complete dryness. The material was often sticky, sometimes almost caramely, most likely because of the high sugar content of this material. In attempt to eliminate one step in the fractionation procedure a preliminary experiment was performed in which RSBM was extracted with 20% acetic acid instead of the usual water extraction followed by acidification. This resulted in a lyophilized supernatant product which was not sticky and caused essentially 100% growth inhibition. However, some uncertainty as to the cause of growth inhibition remained because (a) even after lyophilization both the supernatant and precipitate

fractions had strong acetic acid odors, and (b) the mice ate very little of the precipitate diet.

Thus an experiment was conducted to determine the effect of acetic acid on growth of mice. This was determined by extracting 75 g of HRSBM with one liter of 20% acetic acid four hours, centrifuging and lyophilizing the supernatant. Several other treatments to the acetic acid extract of RSBM were also tested in this experiment. These included the effects of (a) dialysis, (b) heating at both acidic and neutral pH and (c) adsorption on DEAE-cellulose.[a] The dialyzed, neutralized sample was prepared by dialysis of the acetic acid supernatant followed by neutralization, and then heating in a boiling water bath for one minute. The DEAE-cellulose adsorption was determined by stirring 50 g DEAE-cellulose with 880 ml acetic acid extract (from 75 g RSBM) for four hours, centrifuging and testing the supernatant for growth inhibitor activity. A loss of growth inhibitor activity was assumed to indicate adsorption on the DEAE-cellulose. The amount of the ion exchange resin used was based on amounts used by Garlich and Nesheim (42).

The responses by mice when fed these preparations involving acetic acid extraction of RSBM are shown in Table 23. Statistical analyses were performed on only the

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[a]Diethylaminoethyl cellulose, Cellex-D, Calbiochem, Los Angeles, Calif.

TABLE 23.--Growth responses to acetic acid treatments of RSBM.

Dietary Treatment[1]	Wt. gain	Pancreas wt	Feed Efficiency[2]	SBTI	Growth Inhibition		
	g/day	% of body wt		units/g diet	%[3]	% corrected for intake[4]	corrected %[8]
1. HRSBM (22)[5]	0.68[a6]	0.61	0.24	0	---	---	---
2. RSBM (13)	-0.13[e]	0.77	-0.06	6500	119	83	83
73. HRSBM, HOAc-super[9] (8)	0.45[bc]	0.69	0.14	0	38	35	35
69. HOAc-super (15)	0.20[cd]	0.69	0.07	460	70	68	55
72. Dialyzed HOAc-super (8)	0.52[ab]	0.74	0.17	270	28	25	25
74. HOAc-super, heated 1 min (7)	0.26[cd]	0.77	0.09	110	65	65	42
75. Dial. HOAc-super, neutralized, heated 1 min (7)	0.33[bcd]	0.79	0.13	180	55	52	52
76. HOAc-super, DEAE-cellulose super (7)	0.37[bcd]	0.71	0.14	---	50	47	17
68. HOAc ppt (7)	0.13[d]	0.62	0.20	0	78	21	19
$S_{\bar{x}}$[7]	0.07						

[1]Each diet fed four days.

[2-7]See footnotes 2-7, respectively, Table 13.

[8]Corrected % adjusts % corrected for intake to the proper control, either diet 1 or diet 73 (see text for explanation).

[9]HOAc represents acetic acid.

weight gains. Acetic acid accounts for half of the growth depression achieved in the acetic acid supernatant fraction (35% and 68% for diets 73 and 69, respectively, when compared to HRSBM). Therefore, for calculating the growth inhibitor activity in fractions containing considerable acetic acid (i.e. diets 69, 74, 76 and 68) diet 69, the acetic acid extract of HRSBM, must be considered the positive control rather than diet 1. About one-half of the growth inhibition was removed by dialysis as indicated by 55% vs 25% growth inhibition for diets 69 and 72, respectively. Growth inhibitor activity was not destroyed by heat when in acetic acid solution or in a neutral solution (diet 69 vs 74 and 75, $P > 0.10$), but trypsin inhibitor was reduced by the process. Most, if not all of the growth inhibitor was retained on DEAE-cellulose (diets 76 < 73 at $P > 0.10$ and 76 > 69 at $P < 0.10$) which agreed with results previously reported by others (42).

I. Heat Treatments on Different Fractions

Autoclaving RSBM or any of its fractions destroys the growth inhibitory activity of the fraction; however, heating dry RSBM or heating a solution containing the RSBM fraction may not destroy the growth inhibitory activity (109). This was verified in several experiments which involved heating dry RSBM or heating RSBM fractions under different conditions of time and pH. A lyophilized

pH 4.4 supernatant sample was also autoclaved and fed to mice as a check on the heat-lability of this fraction.

The results of this experiment are shown in Table 24. Many of these treatments were also reported in other sections of Experiment 3; therefore, no statistical indications are shown in Table 24. Dry heating RSBM did not improve growth rates of mice confirming the results of Osborne and Mendel (110). Autoclaving the pH 4.4 supernatant destroyed the growth inhibiting as well as its trypsin inhibiting ability of RSBM (diet 71). Heating the pH 4.4 supernatant solution in 15 ml quantities in test tubes placed in a boiling water bath for one or five minutes also destroyed the sample's growth inhibiting activity and about three-fourths of its trypsin inhibiting capability (diets 62 and 63). This indicated that the growth inhibitor was destroyed by mild acid hydrolysis. However, similar heating of an acetic acid extract of RSBM (diet 74) destroyed little if any growth inhibitor activity. Thus the lability of the growth inhibitor(s) under mild acid hydrolysis situations remains uncertain. The growth inhibitor activity of a RSBM fraction in solution at neutral pH was not destroyed by this type of heat treatment even when continued for as long as 10 minutes (diets 8, 18, 19, 20 and 75). Pancreas weights, expressed as per cent of body weight, decreased (except on diet 74) slightly

TABLE 24.--Growth responses of mice when fed various heat treated preparations of raw soybean meal fractions.

Dietary Treatment[1]		Wt gain	Pancreas wt		Feed Efficiency[2]	SBTI	Growth Inhibition	
		g/day	mg	% of body wt		units/g diet	%[3]	% corrected for intake[4]
36.	Dry heated RSBM (4)[5]	-0.13	86	0.75	-0.10	---[6]	116	43
61.	pH 4.4 super (7)	0.20	81	0.70	0.08	2300	66	66
62.	Heated 1 min. (7)	0.56	67	0.53	0.20	670	3	3
63.	Heated 5 min. (7)	0.61	76	0.57	0.23	600	0	0
71.	Autoclaved (8)	0.67	78	0.58	0.19	0	7	7
69.	Acetic acid super (15)	0.20	78	0.69	0.07	460	56	55
74.	Heated 1 min. (7)	0.26	91	0.77	0.09	110	42	42
75.	Neut., dial., heat 1 min. (7)	0.33	96	0.79	0.13	180	55	52
6.	Dial. whey solr. (3)	0.52	130	0.99	0.14	915	42	34
8.	Heated 5 min. (6)	0.26	115	0.85	0.11	192	71	56
12.	Acetone ppt'd W.S. (5)	0.71	108	0.85	0.24	1150	34	33
18.	Heated 1 min. (5)	0.51	96	0.69	0.16	640	56	52
19.	Heated 5 min. (5)	0.84	99	0.74	0.24	670	27	27
20.	Heated 10 min. (5)	0.74	111	0.81	0.21	570	36	36

[1]Many of these dietary treatments are also listed elsewhere in the results part of this thesis.

[2-5]See footnotes 2-5, respectively, Table 13.

[6]Not determined.

in response to heat treatment ($P > 0.10$) probably in response to the decrease in SBTI in the test fraction.

J. Effect of Dialysis on Growth Inhibition

Several experiments were conducted to determine the effects of dialysis on growth inhibitor activity of various fractions. The fractions dialyzed were (a) the 20% acetic acid extract of RSBM, (b) the pH 8.0 supernatant (soybean whey solution) and (c) the acetone precipitated whey solution.

The results reported in Table 25 are the means obtained from several experiments most of which are more completely reported in previous tables. One-half of the growth inhibitor activity was removed or destroyed by dialysis. More than half was removed by dialysis of the acetic acid and pH 8.0 supernatants (55% and 62%, respectively), but less than half (35%) was removed by dialysis of the acetone precipitated whey solution. This may merely reflect normal variation in response but may also mean that acetone causes some destruction of growth inhibitor activity. Some growth inhibitor activity may also be destroyed with time in the acetic acid solution. Trypsin inhibitor was too large (14,000 to 24,000 molecular weight, Table 3) to be removed by dialysis; however, 40% of the SBTI activity present in the acetic acid supernatant was not present after dialysis. This probably indicated acid

TABLE 25.--Effect of dialysis on SBTI and growth inhibitor activities.

Fraction	SBTI	Growth Inhibitor
	(units/g diet)	(units[2])
Acetic acid super (15)[1]	460	55
Dialyzed acetic acid super (8)	270	25
Whey solution (14)	2620	82
Dialyzed whey soln (14)	2110	31
Acetone ppt'd whey soln (7)	870	50
Dialyzed APWS (12)	860	32

[1]Number in parenthesis represents number of animals.

[2]One unit equals 1% growth inhibition corrected for feed intake as described under footnote 4, Table 13.

denaturation especially since total SBTI activity was lower in the acetic acid supernatant than in either the whey solution or acetone precipitated whey solution.

K. Effect of Methionine Supplementation

D-L Methionine was added at the expense of cerelose to both the HRSBM and the RSBM diets at a level of 0.5% of the total diet to determine its effects on growth rates. The results shown in Table 26 indicate that growth rates on the RSBM diet were increased only slightly ($P > 0.05$). The major response to methionine supplementation on this diet was increased feed consumption with no decrease in intake corrected growth inhibition. Methionine supplementation actually decreased growth rates ($P > 0.05$) on the HRSBM diet, but this was partially attributed to decreased feed consumption.

L. Separation on Sephadex G-50.

The results of experiments previously reported, indicated that the factor(s) responsible for much of the growth inhibitor activity of RSBM may be of small molecular weight. The best evidence for this conclusion was the removal of half of the growth inhibitor activity by dialysis. The lack of distinct separation at the various steps in the general fractionation scheme (section B) or in the $(NH_4)_2SO_4$ and bentonite-celite treatments (section F), and the inability to eliminate the growth inhibition

TABLE 26.--Effect of methionine supplementation on growth rates of mice.

Dietary Treatment[1]	Wt. gain	Pancreas wt		Feed Efficiency[2]	SBTI	Growth Inhibition	
	g/say	mg	% of body wt		units/g diets	%[3]	% corrected for intake[4]
1. HRSBM (7)[5]	0.99[a6]	100	0.60[b]	0.29	0	---	---
77. HRSBM + 0.5% met (8)	0.74[a]	86	0.55[b]	0.28	0	25	19
2. RSBM (7)	-0.12[b]	92	0.86[a]	-0.06	6500	112	65
78. RSBM + 0.5% met (8)	0.08[b]	108	0.89[a]	0.03	6500	92	73
$S_{\bar{x}}$[7]	0.09	4	0.04	0.03			

[1]Diets fed 5 days.

[2-7]See footnotes 2-7, respectively, Table 13.

by most heat treatments (section I) also provided indirect evidence that a fairly small molecular weight compound might be involved. Therefore, ion exclusion chromatography techniques were employed in efforts to separate components from extracts of RSBM on the basis of molecular weight (MW). Sephadex G-50[a] was chosen as the gel to use for separation based on its theoretical exclusion limit of approximately 30,000 MW for proteins. With this technique hemagglutinins of 96,000 MW should be excluded and move through the column with the void volume. Trypsin inhibitors should be slightly retained while smaller compounds should be extensively retained.

The pH 4.4 supernatant was the fraction chosen for separation on the G-50 column. This was prepared as previously with a slight modification. Seventy-five g of RSBM were extracted with one liter of distilled water for four hours and centrifuged. The supernatant was acidified to pH 4.4 with concentrated formic acid, centrifuged, and the resulting supernatant immediately neutralized with concentrated NH_4OH. This supernatant (950 ml) was then applied to a G-50 column (8.02 x 109 cm) and eluted with distilled water at a flow rate of 9 ml/minute. The effluent was collected in 50 ml aliquots and selected aliquots assayed for protein, trypsin inhibitor and chymotrypsin inhibitor. The elution pattern is

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[a]Pharmacia Fine Chemicals, Inc., Piscataway, N.J.

illustrated in Figure 3. The effluent in tubes numbered 11 to 45, 46 to 75 and 76 to 137 were combined into three fractions designated I, II and III, respectively, lyophilized and fed to mice for the growth inhibitor assay. Protein was determined by Waddell's method (147) as illustrated in Figure 3 and by measuring absorbance at 280 mμ and 260 mμ (38). Calculated protein values by both methods were identical for peaks I and II (Figure 3), but were higher and somewhat erratic in fraction III when calculated from 280 and 260 mμ absorbancies. Absorbancies at 260 mμ were higher than at 280 mμ in fraction III while the opposite was true in fractions I and II.

The results of the growth inhibitor assays on the fractions from the pH 4.4 supernatant separated on Sephadex G-50 are shown in Table 27. Modifying the pH 4.4 supernatant preparation procedure such that it was acidic for only a short period of time and then neutralized appeared to result in a more efficient retention of growth inhibitor activity. Growth inhibition on diet 70 was 94% as opposed to 44% to 66% (see Tables 14, 23 and 24) in previous experiments. Growth rates and feed efficiencies of mice fed fraction I were not significantly less ($P > 0.10$) than those of mice fed the positive control diet (HRSBM). Therefore, although hemagglutinin assays were not performed, the lack of growth inhibition in the fraction where they should appear indicated that they were not involved in

Figure 3.--Chromatography of the pH 4.4 supernatant on a Sephadex G-50 column. Column dimensions: 8.02 x 109 cm. Sample: 950 ml of pH 4.4 supernatant from RSBM. Eluting buffer: distilled water. Flow rate: 9 ml per minute. Protein (o—o), SBTI (•—•), SBChTI (Δ—Δ).

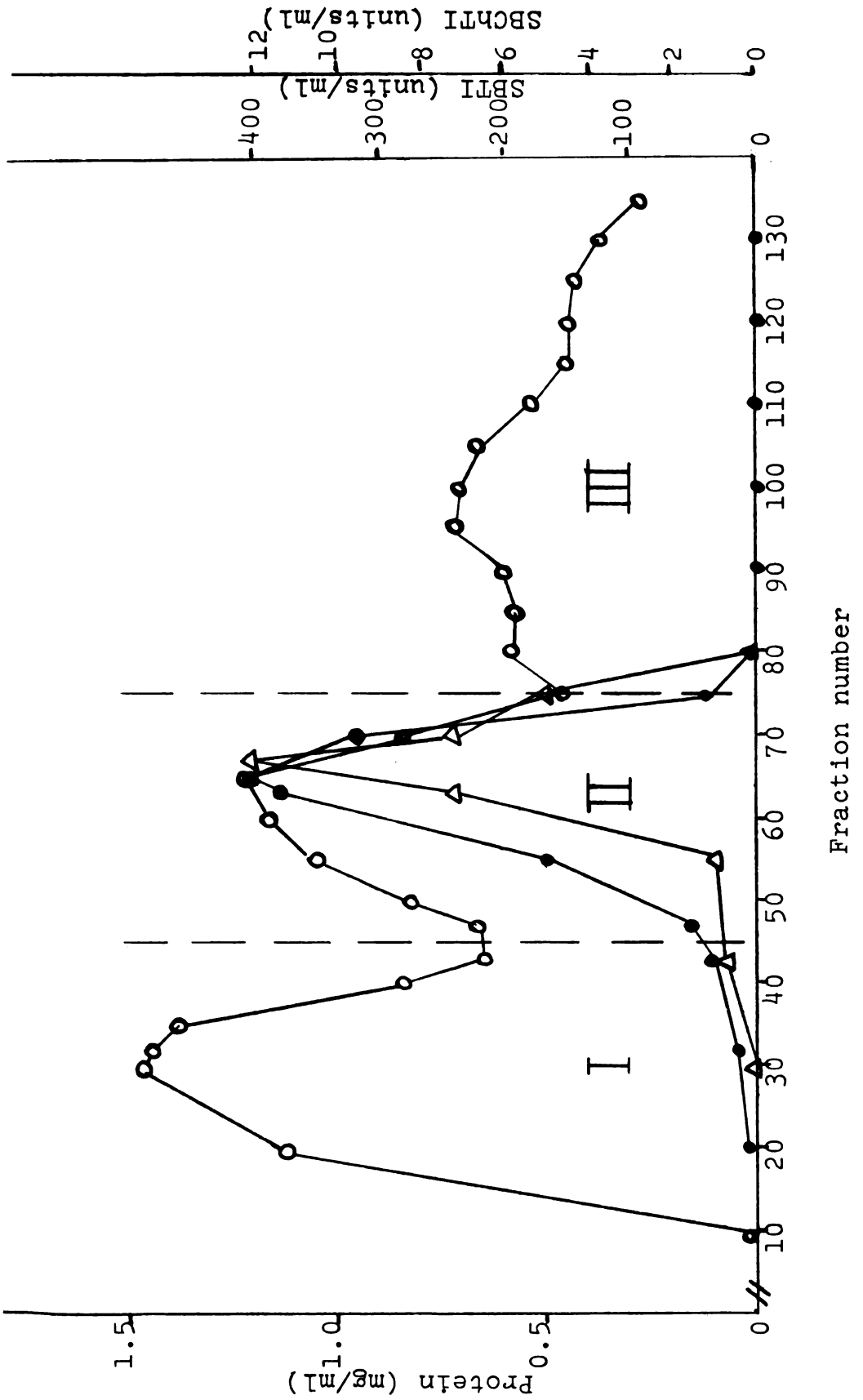

Protein (mg/ml)
SBTI (units/ml)
SBChTI (units/ml)
Fraction number
I
II
III

TABLE 27.--Growth inhibitor assay of fractions of pH 4.4 supernatant separated on a Sephadex G-50 column.

	Dietary Treatment[1]	Wt. gain	Pancreas wt		Feed Efficiency[2]	SBTI	Growth Inhibition	
		g/day	mg	% of body wt		units/g diet	%[3]	% corrected for intake[4]
1.	HRSBM (7)[5]	0.99[a][6]	100[a]	0.60[c]	0.29[a]	0	---	---
2.	RSBM (7)	-0.12[c]	92[ab]	0.86[a]	-0.06[c]	6500	112	65
79.	pH 4.4 super (7)	-0.03[c]	74[b]	0.63[c]	-0.01[c]	2900	103	94
80.	G-50, Fraction I (7)	0.84[a]	112[a]	0.71[bc]	0.30[a]	17	15	12
81.	G-50, Fraction II (7)	0.51[b]	108[a]	0.77[ab]	0.17[b]	2000	48	42
	$S_{\bar{x}}$[7]	0.09	7	0.04	0.03			
1.	HRSBM (8)	0.63[a]	89	0.63	0.21[a]	0	---	---
82.	G-50, Fraction III (7)	0.40[b]	80	0.60	0.13[b]	4	37	38
	$S_{\bar{x}}$[7]	0.06	6	0.04	0.02			

[1]Diets fed five days.

[2-7]See footnotes 2-7, respectively, Table 13.

growth inhibition. The growth inhibitor activity present in the pH 4.4 supernatant was about equally divided between fractions II and III. Fraction II, which contained the SBTI, caused pancreatic enlargement (expressed as per cent of body weight) while fraction III did not. Pancreases were also slightly enlarged in mice fed fraction I.

The growth inhibition attributed to fraction II may have been caused by (a) SBTI, (b) another compound, probably proteinaceous, with a MW similar to that of the trypsin inhibitors or (c) contamination of the growth inhibitor present in fraction III. Possibility c cannot be ruled out at this time since the large volume of sample applied to the G-50 column did not allow for complete separation of the peaks (Figure 3); therefore, a considerable amount of overlap of components in fractions II and III was possible.

The reduced growth rates and feed efficiencies with no increase in pancreas size attributed to fraction III indicated that a growth inhibitor in RSBM had been separated from trypsin inhibitors and pancreatic enlargement factors. The retention time on a G-50 column indicated that the fraction III growth inhibitor was a fairly small compound, definitely smaller than SBTI. However, since fraction III contained essentially all of the small MW water and acid soluble components present in RSBM, a considerable amount of material, probably mostly extraneous, was still present in this fraction.

The presence of carbohydrate in fraction III was confirmed by a positive response to the Molish test (38), a qualitative test for the presence of carbohydrate. This was expected because of the previously discussed (see section H) stickiness associated with many of the lyophilized supernatant fractions. However, further purification is needed to ascertain whether a carbohydrate is related to growth inhibition or if it is merely an unrelated contaminant still present in fraction III.

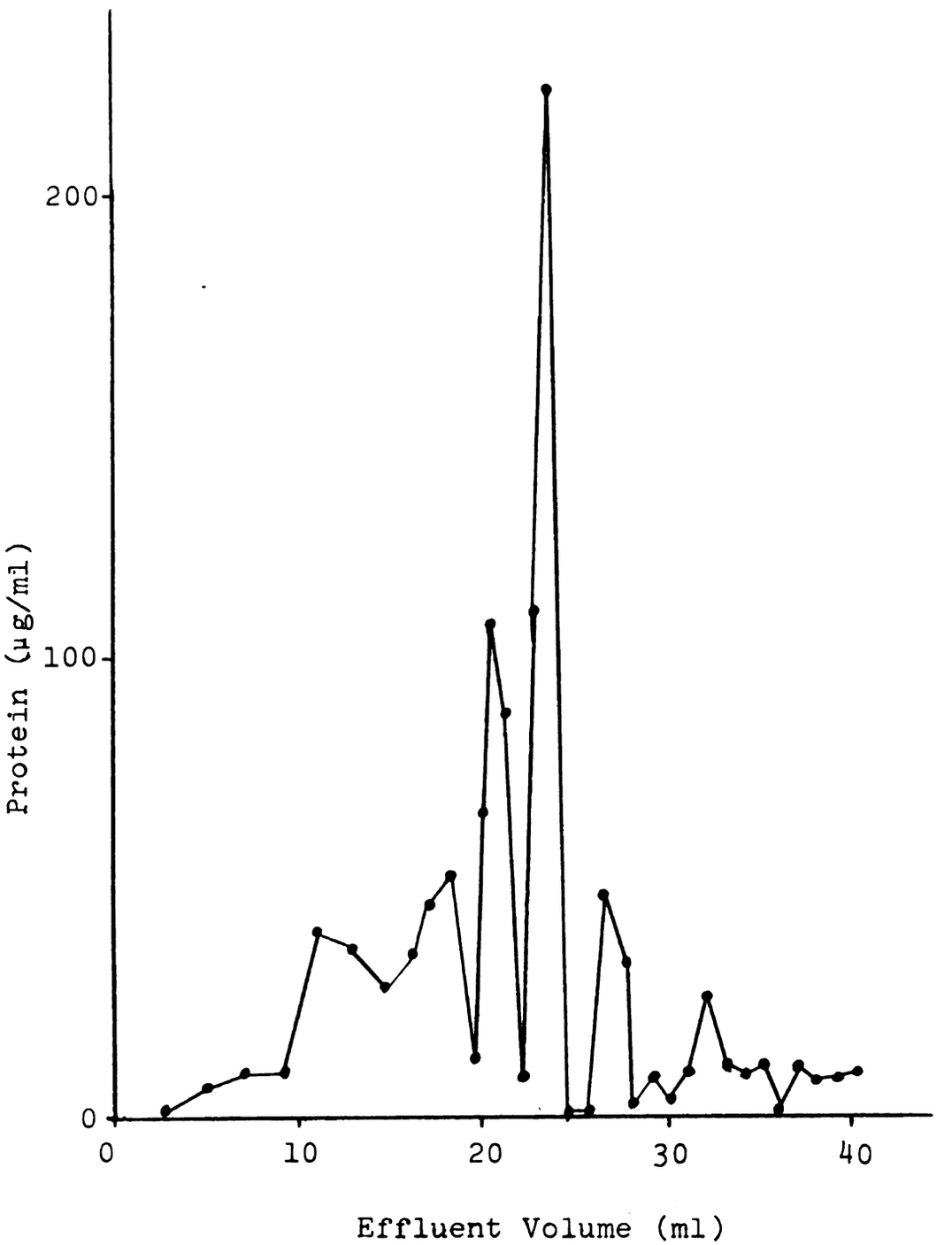
M. Further Characterization of G-50, Fraction III

Several experiments were performed to further characterize the growth inhibitor present in fraction III. These included chromatography on a Sephadex G-25 column, electrophoresis and heat treatments.

Figure 4 illustrates the elution pattern of the G-50 fraction III using a Sephadex G-25 column. Two major protein peaks were observed as well as indications of two to four minor peaks. Only a minor peak was observed eluting with the void volume (11 ml) and this possibly represented contamination by larger proteins from fraction II. The retarded elution rates of most of the fraction III proteinaceious material on G-25 indicated molecular weights of less than 5000. No protein could be detected after polyacrylamide-gel electrophoresis[a] of fraction III

[a]The polyacrylamide-gel electrophoresis determinations were made by Dr. J. E. Wilson, whose assistance is gratefully acknowledged.

Figure 4.--Chromatography of the G-50 fraction III on a Sephadex G-25 column. Column dimensions: 0.92 x 41.5 cm. Sample: 20 mg of G-50 fraction III in 1 ml of distilled water. Eluting buffer: distilled water. Flow rate: 5.6 ml per hour.


200
100
0
Protein (µg/ml)
0
10
20
30
40
Effluent Volume (ml)

indicating either reaction with the gel or diffusion. Diffusion would indicate a very small molecule, possibly 1000 MW or less; therefore, confirming the G-25 elution results.

High voltage electrophoresis was performed on the G-50 fraction III and the first four peaks separated on the G-25 column according to the procedure of Ryle et al. (123). Insufficient material was available from peaks 5 and 6 (27 and 32 ml, respectively) for electrophoresis. Electrophoresis was carried out in a pyridine-acetic acid-water buffer at pH 3.5 for two hours at 2000 volts using a High Voltage Electrophorator Model D.[a] Sample components were identified by staining using ninhydrin reagent for proteins and 0.5% sodium periodate followed by 0.5% benzidine sprays for carbohydrates. The results are illustrated in Figure 5. As many as eight positively charged protein bands were identified. One positively charged band was identified with the carbohydrate staining system and its position corresponded with that of protein band C possibly indicating a glycopeptide. Chromatography on Sephadex G-25 did not selectively segregate any particular charged component into a particular peak, but the concentrations of some components appeared to vary with peaks. Band C was most intense in G-25 peak 2 and least intense in peak 4.

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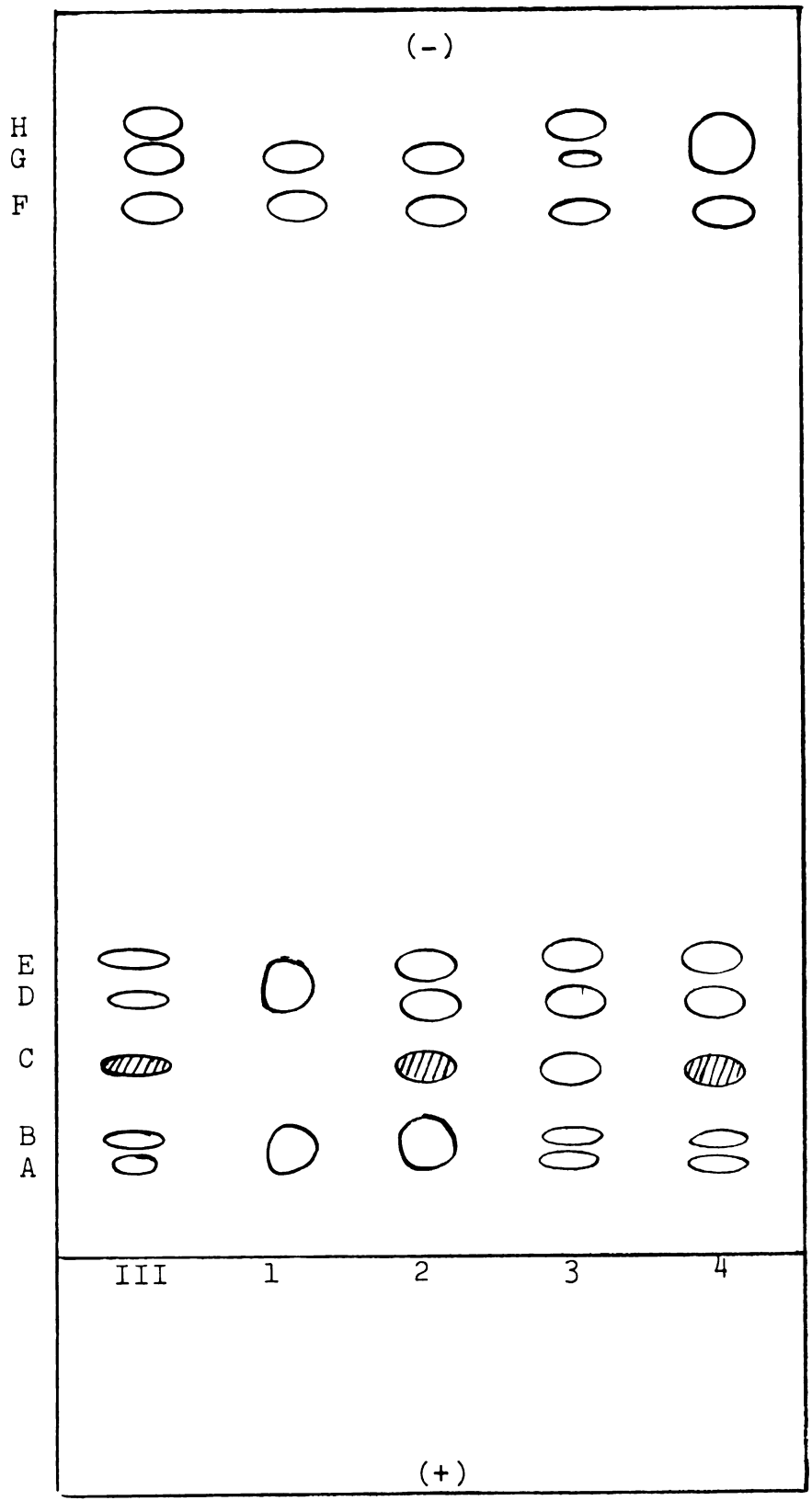
[a]Gilson Medical Electronics, Middleton, Wisc.

Figure 5.--High voltage electrophoresis, pH 3.5, of G-50 fraction III and the first four peaks separated on a G-25 column. (III) fraction III, (1, 2, 3, 4) peaks 1, 2, 3 and 4, respectively, from the G-25 column separation of fraction III (Figure 4). (⬭) protein stain, (▨) protein and carbohydrate stain.


(-)
H
G
F
E
D
C
B
A
III
1
2
3
4
(+)

Protein band E was most intense in peak 3 while bands D and F were most intense in peak 4.

If band C is a glycopeptide and is responsible for growth inhibition, then growth inhibitor activity in fraction III should be destroyed by mild acid or base hydrolysis. Previous heat treatments indicated that growth inhibitor activity may be destroyed by mild acid hydrolysis (section I). In order to test this hypothesis, a solution containing G-50 fraction III was acidified to pH 4.2 with 6 N HCl, heated in 15 ml capacity test tubes in a boiling water bath for 10 minutes, rapidly cooled and lyophilized. Growth inhibitor assays and electrophoresis were performed on this mild acid hydrolyzed fraction III material.

The growth inhibitor activity of fraction III was not destroyed by the mild acid hydrolysis treatment used as shown in Table 28. Four mice each were fed diets 79, 89 and 82 as checks on previous responses to the materials in these diets. Diet 90 contained a mixture of G-50 fractions I and II because in later preparations of fraction III on the large G-50 column, no efforts were made to obtain complete separation between fractions I and II; however, smaller quantities were put on the column and fraction III was completely separated from fraction II. The growth responses to the pH 4.4 supernatant and the G-50 fraction III were identical to previous results. However, the fraction containing SBTI (diet 90) caused no

TABLE 28.--Growth inhibitor assay of the mild acid hydrolyzed G-50 fraction III and of some other pH 4.4 supernatant fractions.

Dietary Treatment[1]	Wt. gain	Pancreas wt		Feed Efficiency[2]	SBTI	Growth Inhibition	
	g/day	mg	% of body wt		units/g diet	%[3]	% corrected for intake[4]
1. HRSBM (7)[5]	0.69[a][6]	86[b]	0.68[c]	0.25[a]	0	---	---
2. RSBM (6)	-0.21[d]	60[c]	0.80[b]	-0.15[d]	6500	130	65
79. pH 4.4 super (4)	0.02[c]	82[b]	0.89[ab]	0.01[c]	2900	97	97
89. G-50, Fractions I & II (4)	0.75[a]	129[a]	0.97[a]	0.27[a]	2100	0	0
82. G-50, Fraction III (4)	0.36[b]	---	---	0.13[b]	4	48	48
88. Mild acid hydrolyzed (7) G-50, fraction III[8]	0.30[b]	67[b]	0.64[c]	0.11[b]	---	57	57
$S_{\bar{x}}$[7]	0.06	6	0.04	0.02			

[1]Diets fed six days, three days for diet 82.

[2-7]See footnotes 2-7, respectively, Table 13.

[8]Solution acidified to pH 4.2 and heated in boiling water bath for 10 minutes.

growth inhibition but did cause significant pancreatic enlargement.

Electrophoresis of the mild acid hydrolyzed fraction III showed no change in the mobility of seven previously detected protein bands, including the carbohydrate-staining band, but showed elimination of the fastest moving positively charged peptide (H) and the appearance of a slightly negatively charged peptide (Figure 6). Thus, the growth inhibitor must be in one of the seven (only four distinctly separate on Figure 6) unchanged portions, only one of which is possibly a glycopeptide. This lack of change in the electrophoretic pattern agreed with the growth inhibitor results since the mobility of the only band which could possibly be a glycopeptide was not changed by the acid heat treatment used. To further test the growth depressing activity of this fraction, acid and base hydrolysis at pH 2 and pH 11 using similar heat treatments should probably be performed before a glycopeptide is definitely eliminated from consideration.

Samples of HRSBM, RSBM, pH 4.4 supernatant, G-50 fractions I, II and III, and hydrolyzed fraction III were assayed for hemolytic activity by the procedure of M. Jones.[a] Hemolytic activity was used as an indication of the presence of saponins which are known to be present in

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[a]Unpublished procedure used as a screening test for saponins primarily in alfalfa, Crop Science Department, Michigan State University.

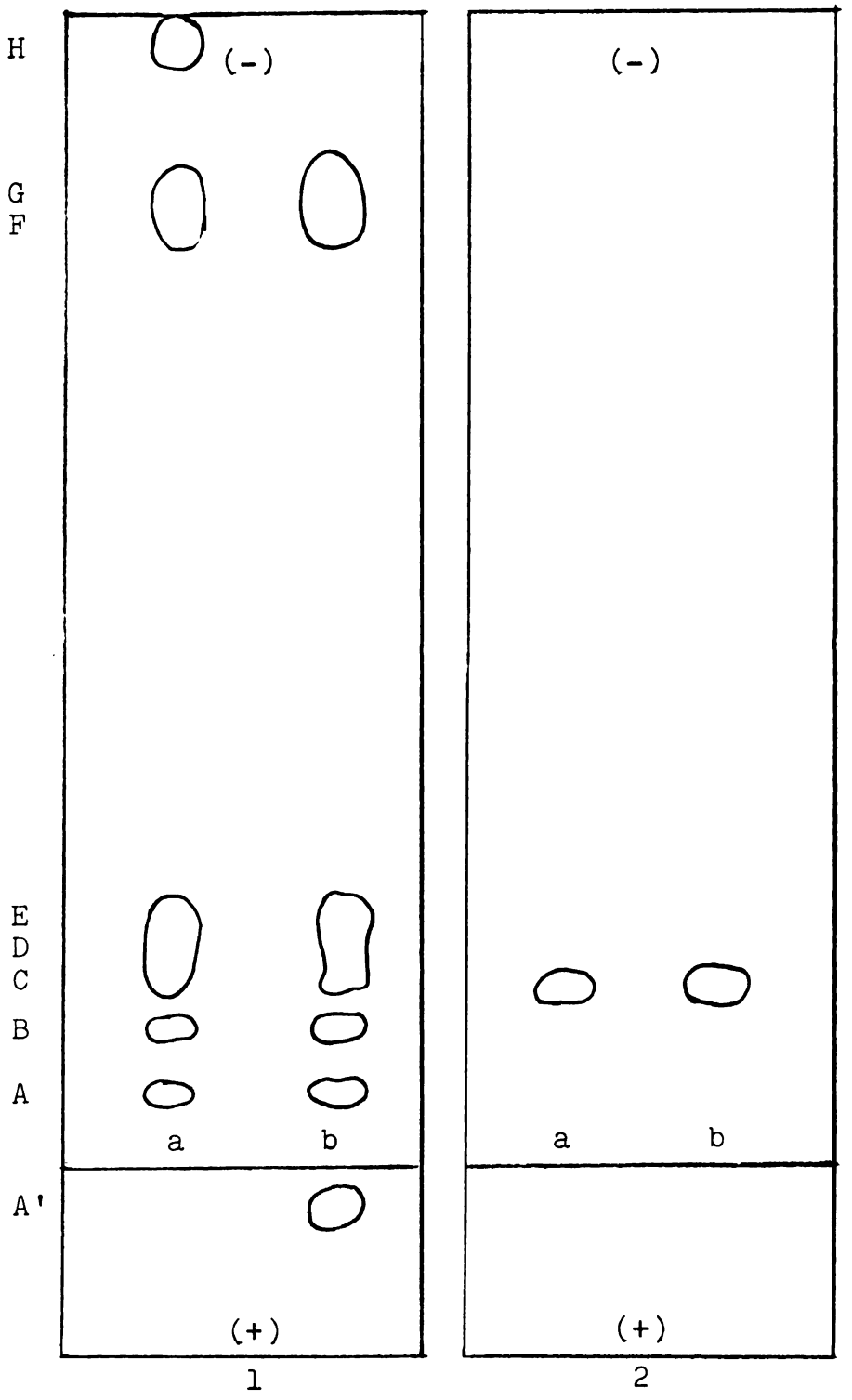

Figure 6.--High voltage electrophoresis, pH 3.5, of (a) fraction III and (b) mild acid hydrolyzed fraction III. (1) ninhydrin stain, (2) periodate-benzidine stain.

soybeans (152) but probably do not affect growth rates of animals (12, 66). No hemolytic activity was detected in any of the samples tested.

Table 29 summarizes the extent of purification of the fraction III growth inhibitor. When growth inhibitor activity is expressed on a dry matter basis, the purification is only four-fold since the G-50 fraction III still contains a considerable amount of water and acid soluble small MW compounds. However, when activity is expressed on a protein basis, which will be the appropriate comparison if final purification shows it to be a protein, then purification is more than 28-fold. The water-insoluble residue accounts for nearly 40% of the protein present in RSBM, the pH 4.4 precipitate is essentially pure protein and more than 60% of the protein in the pH 4.4 supernatant is accounted for by G-50 fractions I and II.

N. Integrated Discussion of Soybean Growth Inhibitor Isolation and Characterization Experiments

Mice responded rapidly to the dietary treatments. The average daily gain after three days on a diet was essentially the same as after four to six days. Additional days on a diet did provide a slight reduction in variance within the dietary groups. Four mice were used per dietary treatment in early experiments, but to reduce the standard error, five to seven were used whenever sufficient test material was available in later experiments.

TABLE 29.--Purification of fraction III growth inhibitor.

Fraction	Growth Inhibitor Activity		
	units[1]/g DM	units[1]/g protein	% yield
RSBM	1.54	3.00	100
Water extract	3.90	7.00	95
pH 4.4 supernatant	4.40	33.80	71
Sephadex G-50, fraction III	6.06	86.00	57

[1]One unit equals 1% growth inhibition corrected for feed intake as described under footnote 4, Table 13.

The per cent growth inhibition corrected for feed intake was used as the index of growth inhibitor activity in order to correct for differences in weight gains from one experiment to the next and to correct for inequalities in feed consumption. The correction for differences in feed consumption on the test versus the control diet was usually small except for mice fed the RSBM diet. Consumption was consistently low on the RSBM diet and consequently calculated growth inhibition was less precise on that diet since it was somewhat confounded with starvation effects. The data summarized in Table 30 illustrate the overall mean and ranges of means for both average daily gains and growth inhibitor activities achieved on several diets which were tested at different times during these trials. This summary illustrates very good precision and repeatability with this growth assay. The range was more for actual weight gains among trials than when gains were expressed as a percentage of that particular trial control group. Much of the variation in rate of gains among experiments was due to changes in environmental conditions. For instance, weight gains on all groups were proportionately lower in feeding trials conducted during hot, humid summer weather.

The results of these experiments indicate that there may be two or more factors in RSBM which inhibit growth. The two primary factors are in the water-insoluble residue and in the G-50 fraction III. Trypsin inhibitors and other

TABLE 30.--Overall mean and range in means of weight gains and growth inhibition achieved on several diets.

Dietary Treatment	Means of wt gain		Means of Growth Inhibition	
	Overall mean	Range	Overall mean	Range
	g/day		% corrected for intake	
HRSBM (12)[1]	0.82	0.58 to 1.16	---	---
RSBM (8)	-0.21	-0.39 to -0.10	73	55 to 96
Water-insoluble residue (3)	0.55	0.44 to 0.73	43	38 to 49
Dialyzed whey soln (3)	0.61	0.40 to 0.74	31	28 to 34
Acetone ppt'd WS (2)	0.61	0.51 to 0.71	36	33 to 40

[1]Number of trials.

factors may also affect growth, but the extent of their effect on growth is not as well substantiated.

The growth-inhibition attributed to the water-insoluble residue may be caused by the same factor present in the soluble portion. In such a case, the growth inhibitory factor would be a relatively insoluble compound. The poor efficiency of water extraction and the incomplete separation of growth inhibitor at several precipitation steps may indicate such a situation. However, if the growth inhibition attributed to the water-insoluble residue is merely a reflection of incomplete dissolving, re-extracting or extending the extraction time should reduce the growth inhibitor activity of the more completely extracted residue. This did not occur. Digestion by gastrointestinal enzymes also failed to remove or destroy the growth inhibiting properties of the water-insoluble residue. Thus it appears that the residue may be (or contain) a growth inhibitor completely separate from the inhibitors in the soluble portion of RSBM.

The water-insoluble residue may accomplish its growth inhibiting effects via its indigestible and insoluble properties. Decreased protein digestion is generally accepted as a major factor in explaining the growth inhibition caused by raw soybeans. Digestibility studies indicate that RSBM contains a fraction which becomes digestible only after heating (80, 85, 106). The steam cooking

necessary to bring about such a response causes a loss in cystine with a corresponding appearance of lanthionine and liberated sulfide as well as increased susceptibility to enzymatic hydrolysis (1). Fractionation of RSBM showed that these properties were attributed to the water-insoluble residue (1).

There is considerable evidence to indicate that a small molecular weight compound accounts for much of the growth inhibition caused by RSBM. Retardation on Sephadex-G-50 and G-25 columns, loss of activity by dialysis and lack of detection by polyacrylamide-gel electrophoresis all strongly indicate a small molecule. Incomplete separation of growth inhibitor into either precipitate or supernatant in many of the fractionation steps also indicates that a small molecule may be involved. Movement in an electric field and adsorption on DEAE-cellulose indicate that the molecule carries a charge, which is most likely positive. Therefore, it may be reasonable to assume that this small, charged molecule can readily become trapped by or attached to larger protein molecules and in this way be incompletely separated by a particular chemical treatment. Such a situation may explain the partial growth inhibitor activity of the pH 4.4 and pH 8.0 precipitates and the inconsistent separation of growth inhibitor activity by $(NH_4)_2SO_4$ fractionation. Other research teams have also observed this slight reduction in growth rate of

rats (117) or chicks (42) fed diets containing pH 4.4 and pH 8.0 precipitated fractions, but tended to ignore this phenomena since they were trying to correlate growth inhibition with trypsin inhibitor activity.

The attachment of a small molecule to a larger protein may also explain the growth inhibition attributed to trypsin inhibitors, even five times crystallized SBTI. Eldridge et al.(35) detected 6 to 13 bands or polyacrylamide-gel electrophoresis from each of 9 commercial SBTI samples. This indicates that crystallinity is no guarantee of purity and may also account for the disagreement in literature over the growth-inhibiting properties of SBTI.

Pancreas enlargement appeared to be more closely related to dietary SBTI activity than to growth inhibition. In experiment II, this correlation between pancreas enlargement and SBTI activity was very obvious. Although there were discrepancies in some of the mouse growth assays, pancreas enlargement was usually closely related to SBTI activity. One of the best illustrations of this was the second Sephadex G-50 separation experiment. In that experiment, the SBTI-containing diet was the only one which caused pancreatic enlargement. The diet causing half of the growth inhibition (fraction III) caused no pancreas enlargement. On the other hand, pancreases were not significantly enlarged when crystalline SBTI was added to diets; however, the level may have been too low to have a marked effect.

Differences in actual pancreas size attributed to dietary treatments were generally only moderate and statistically significant differences usually occurred only when pancreas size was corrected to a constant body weight. This moderate effect on pancreas size may be attributed to the short duration of the mouse growth trials since Rackis (113) found that rats required nine days to reach optimum pancreas enlargement. When growth trials were continued for 21 days as with the rats in Experiment II, the actual pancreas size of SBTI-fed rats was enlarged.

These results do not necessarily imply a cause and effect relationship between SBTI and pancreas enlargement. Several factors have been implicated in pancreatic enlargement including the level of fat in the diet (105), soybean hulls (133) and trypsin inhibitors (43). However, the results of these experiments indicate that pancreatic enlargement may be associated with SBTI activity but not associated with growth inhibitor activity.

The cause of reduced consumption of RSBM diets was explored but not ascertained. Limited attempts to extract a factor responsible for reducing feed consumption were unsuccessful. Almost any treatment to RSBM restored feed intake essentially to that of the HRSBM-fed group. Wada et al. (146) suggested that hemagglutinins elicit their growth inhibiting properties by reducing feed consumption. Feed consumption of mice fed G-50 fraction I, the fraction

most likely to contain hemagglutinins, was slightly less than intakes of the HRSBM-fed group, and in this manor was in agreement with Wada's claims. However, no factor(s) was isolated which sufficiently accounted for all of the reduced intake on the RSBM diet.

CHAPTER V

SUMMARY

Pancreas weight, trypsin and chymotrypsin contents increased directly with body weight in bull calves from birth to one year of age. The new born calf was the only age group which indicated any marked differences from the mean of all age groups. Pancreas weight and trypsin content were slightly less at this age while chymotrypsin content per mg of pancreas tissue was higher than at all other ages studied.

Diets containing soybean trypsin inhibitor (SBTI) caused pancreatic hypertrophy in rats with corresponding increases in trypsin and chymotrypsin activities. Enzyme activities per mg of pancreas tissue remained constant. These same SBTI-containing diets caused increased chymotrypsin activity and stability, decreased trypsin stability but did not change free trypsin activity in the intestinal contents. Intestinal protein digestion, as measured by an in vitro system, was not impaired in the intestinal contents of the SBTI-fed rats. Growth rates were depressed on only two of the four SBTI diets indicated that the growth depression exerted by raw or minimally processed soybean products is not caused by SBTI and apparently occurs by

some mechanism other than by interference with protein digestion.

Rat growth data and literature data indicated that SBTI and growth inhibition activities in soy preparations were not the same compound. Therefore, attempts were made to separate these two factors by partition and precipitation techniques after establishing a growth assay using mice. A small molecular weight growth inhibitor present in unheated soybean meal was separated from SBTI by ion exclusion chromatography on a Sephadex G-50 column and partially characterized. This growth inhibitor decreased weight gains and feed efficiencies of mice without causing pancreatic enlargement. Further evidence of the small size of this growth inhibitor was its retardation on a Sephadex G-25 column, removal by dialysis and lack of detection by polyacrylamide-gel electrophoresis. Movement toward the cathode under high voltage electrophoresis at pH 3.5 and apparent adsorption on DEAE-cellulose indicated a positively charged compound at that pH.

Other factors in soybeans may also inhibit growth. The water-insoluble residue accounted for about 40% of the growth inhibitor activity of raw soybean meal. This growth inhibition could not be removed or destroyed by gastrointestinal enzyme digestions or by several other solvents tested. Fractions containing SBTI generally caused pancreatic enlargement and had some growth depressing activity.

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APPENDICES

APPENDIX TABLE I.--Body weight and pancreas size, dry matter, and enzyme content of bull calves from birth to 12 months of age.

Age and calf no.	Body wt	Pancreas			
		wt	Dry matter	Trypsin	Chymotrypsin
	(kg)	(g)	(%)	(units/mg DM)	
1 day old					
1	29.0	16.8	23.2	4.73	8.42
2	34.9	20.0	26.0	3.03	5.23
3	44.9	23.2	24.9	5.20	8.87
4	33.6	19.0	25.0	5.30	4.32
5	33.6	14.5	23.4	4.46	8.96
2 months					
175	73.5	44.0	24.0	3.82	2.02
176	76.2	67.0	32.9	6.97	3.75
177	68.0	44.0	21.6	2.76	1.87
178	68.8	68.0	23.0	5.92	3.27
185	74.8	43.0	22.2	5.27	3.55
4 months					
163	137.9	112.0	25.4	5.39	4.65
170	140.6	94.0	23.9	4.71	2.98
171	146.1	121.0	22.4	4.74	4.05
172	135.2	94.0	19.1	2.91	2.65
174	129.7	93.0	25.4	3.66	3.18
5 months					
162	152.4	89.0	23.2	4.73	4.17
6 months					
154	188.7	190.0	33.6	6.46	4.63
155	198.7	174.0	21.4	4.66	3.04
157	163.7	175.0	24.6	5.64	4.15
7 months					
143	222.3	188.0	22.0	3.41	2.90
144	206.4	168.0	24.8	5.69	4.97
145	191.9	153.0	18.3	7.01	5.55
146	193.7	146.0	24.5	3.64	3.24
147	175.5	149.0	22.1	3.27	2.80
8 months					
135	180.5	123.0	22.9	5.13	3.17
136	240.4	186.0	23.1	6.13	4.08
137	235.0	136.0	23.7	5.02	4.48
138	225.9	111.0	22.1	3.82	3.22
142	225.9	174.0	23.3	5.07	4.90
148	217.7	143.0	23.0	5.25	4.66
10 months					
123	276.7	197.0	22.3	4.71	4.32
124	224.5	245.0	23.9	3.73	3.53
127	298.5	200.0	22.8	5.21	3.95
132	299.4	222.0	24.1	9.63	10.09
134	278.5	236.0	22.6	8.32	7.87
11 months					
117	306.2	290.0	21.8	7.18	6.56
118	316.2	226.0	22.1	5.22	4.61
119	313.0	(95.0)	20.9	3.35	3.09
12 months					
101	344.7	238.0	23.5	4.66	4.39
102	328.4	245.0	24.2	6.08	4.91
104	357.4	277.0	22.8	6.37	5.52
107	382.8	314.0	19.6	5.98	4.21
109	289.4	225.0	21.8	6.19	4.72

APPENDIX TABLE II.--Amount of test fractions present in 100 g of test diet.

Diet No.	Fraction	Amount (g/100g diet)
1.	HRSBM (positive control)	50.0
2.	RSBM (negative control)	50.0
3.	Dialyzed whey solution	4.5
4.	Water-insoluble residue	24.1
5.	pH 4.4 ppt	8.1
6.	pH 8.0 ppt	0.4
7.	Acetone ppt'd whey solution	2.4
8.	H_{60} whey solution	2.2
9.	H60 whey solution	2.2
10.	Water-insoluble residue$_3$	25.0
11.	Dialyzed whey solution$_3$	2.9
12.	Acetone ppt'd whey solution$_3$	2.2
13.	APWS - 100% $(NH_4)_2SO_4$ super^3	0.4
14.	APWS - 100% $(NH_4)_2SO_4$ ppt	0.9
15.	APWS - 50% $(NH_4)_2SO_4$ super	0.8
17.	APWS - Bentonite-celite super	0.7
18.	APWS - heated 1 min	1.4
19.	APWS - heated 5 min	1.3
20.	APWS - heated 10 min	1.3
21.	Water-insoluble residue$_4$	22.0
22.	Residue - water super	0.8
23.	Residue - water ppt	21.9
24.	Residue - 0.15 N NaCl super	0.7
25.	Residue - 0.15 N NaCl super	21.5
26.	Residue - pepsin super	0.9
27.	Residue - pepsin ppt	21.5
28.	Residue - papain super	1.9
29.	Residue - papain ppt	21.0
30.	APWS - 50% $(NH_4)_2SO_4$ super	0.6
31.	APWS - 50% $(NH_4)_2SO_4$ ppt	0.4
32.	APWS - bentonite-celite $(NH_4)_2SO_4$ super	0.6
33.	APWS - bentonite-celite $(NH_4)_2SO_4$ ppt	0.6
34.	Dialyzed whey solution$_4$	1.7
35.	Acetone ppt'd whey solution$_4$	0.8
36.	Vacuum heated RSBM	50.0
37.	Dry $CHCl_3$-MeOH extracted RSBM	50.0
38.	pH 2 $CHCl_3$-MeOH extracted RSBM	50.0
39.	pH 10 $CHCl_3$-MeOH extracted RSBM	50.0
40.	Lipase extracted RSBM	46.0
41.	pH 10 $CHCl_3$-MeOH extracted RSBM	22.6
42.	Lipase extracted RSBM	18.8
42 - b.	Lipase digested RSBM - ppt	33.0
43.	0.15 N NaCl extracted RSBM	25.5
44.	1% Triton-X-100 extracted RSBM	22.6
45.	Amylase digested RSBM - ppt	33.0

APPENDIX TABLE II.-- Continued.

Diet No.	Fraction	Amount (g/100g diet)
46.	Pepsin digested RSBM - ppt	23.6
47.	Trypsin digested RSBM - ppt	31.6
48.	Water-insoluble residue	24.2
49.	Lipase digested RSBM - super	18.9
50.	Undialyzed whey solution (pH 8.0 super)	8.8
51.	APWS - 25% $(NH_4)_2SO_4$ ppt	0.3
52.	APWS - 80% $(NH_4)_2SO_4$ ppt	0.238
53.	APWS - 80% $(NH_4)_2SO_4$ ppt 5 x crystalline SBTI	0.238 0.152
54.	5 x crystalline SBTI	0.152
55.	5 x crystalline SBTI, 4 x	0.608
56.	APWS - 80% $(NH_4)_2SO_4$ super	1.87
57.	Amylase digested RSBM - super	26.0
58.	Pepsin digested RSBM - super	20.9
59.	Trypsin digested RSBM - super	16.3
60.	pH 4.4 super	8.3
61.	pH 4.4 super (conc)	12.4
62.	pH 4.4 super (conc) - heated 1 min super	6.5
63.	pH 4.4 super (conc) - heated 5 min super	5.8
64.	Undialyzed whey solution (pH 8.0 super)	5.3
65.	Dialyzed whey solution	3.0
66.	APWS (undialyzed ppt)	2.6
67.	APWS - super	7.1
68.	20% acetic acid treated RSBM - ppt	25.6
69.	20% acetic acid treated RSBM - super	25.1
70.	Water-insoluble residue$_6$	19.5
71.	Autoclaved pH 4.4 super	5.5
72.	Dialyzed acetic acid super	6.8
73.	20% acetic acid extract of HRSBM	14.2
74.	20% acetic acid super, heated 1 min	13.5
75.	Neutralized, dialyzed acetic acid super, heated 1 min.	6.8
76.	Acetic acid super, DEAE-cellulose super	11.5
77.	D-L-methionine (HRSBM + met)	0.5
78.	D-L-methionine (RSBM + met)	0.5
79.	pH 4.4 super	12.7
80.	G-50 fraction I	1.3
81.	G-50 fraction II	1.7
82.	G-50 fraction III	7.7
88.	Fraction III, pH 4.4 - heated 10 min.	6.7
89.	G-50 fractions I and II	1.7

APPENDIX TABLE III.--Individual body weight gains, pancreas size and feed consumption of rats and mice fed 3 soybean diets for 7 days.

Dietary Treatment and Animal No.	Wt. gain		Pancreas wt.		Feed Consumption	
	R[1]	M[2]	R	M	R	M
	(g/day)		(mg)		(g/7 days)	
HRSBM						
1	6.24	0.97	355	133	87	29
2	4.68	0.91	270	134	86	26
3	5.49	0.84	366	84	91	25
4	6.43	1.10	258	119	101	24
RSBM						
1	4.46	-0.40	417	---	70	8
2	4.56	-0.27	470	---	71	7
3	4.21	-0.52	377	---	76	8
Dialyzed Whey Solution						
1	5.79	0.60	455	134	83	22
2	4.09	0.89	488	119	88	24
3	5.17	-0.07	498	110	89	13

[1]R equals rat

[2]M equals mouse.