GROWTH INHIBITION OF RATS FED RAW NAVY BEANS

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY

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1964

This is to certify that the

thesis entitled

Growth inhibition of rats fed raw navy beans

presented by

Madhusudan Laxman Kakade

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biochemistry

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Date September 24, 1964

ABSTRACT

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RAW NAVY BEANS

By Madhusudan Laxman Kakade

The original observation of Everson and Heckert that raw navy beans are "toxic" to the rats and that autoclaving the beans destroyed the toxic effect has been confirmed. Autoclaving the beans also destroyed the trypsin inhibitor and hemagglutinins present in them. The results of feeding navy beans heated for various periods on the growth of rats indicated that heating for 5 minutes at 121° was optimal.

Raw navy beans supplemented with all deficient amino acids did not support the growth of rats. Rats fed autoclaved beans supplemented with methionine alone or with all deficient amino acids grew as well as those fed the casein diet.

Supplementation with methionine and/or vitamin B_{12} failed to correct the growth depression but reduced mortality in rats fed raw navy beans. Antibiotics supplementation prevented weight losses and in combination with methionine and vitamin B_{12} promoted limited growth.

The experimental diet of raw navy beans produced the most severe morphological changes which included growth inhibition, reduced food consumption, pancreatic

acinar atrophy, fatty metamorphosis of the liver, thyroid follicular atrophy and hyperkeratosis of the esophagus and skin. Significant morphological changes were not observed in the organs of rats fed autoclaved bean diet as compared to the control group.

In vitro digestibility studies indicated that the amino acids are released from raw navy beans at a slower rate than those from heat-treated beans. Nitrogen digestibility by rats fed raw bean diet or raw bean diet with added antibiotics is significantly less than that of heated bean diet or heated bean diet with added antibiotics. However, digestibility value greater than that of raw bean diet was observed with raw bean diet supplemented with antibiotics. Methionine digestibility was poor in rats fed raw bean diet and was significantly improved by autoclaving the beans and/or supplementing antibiotics.

The supplementation of trypsin on the growth of rats receiving raw bean diet was ineffective. Moreover, it was found that trypsin was toxic to the rats fed autoclaved bean diet. Preliminary soaking prior to autoclaving did not improve the growth promoting effect of autoclaved navy beans.

Five different fractions were isolated from raw navy beans and their effect on the growth of rats was studied. All these fractions except one, which had the

highest hemagglutinating activity, significantly inhibited the growth of rats.

Growth inhibition of rats fed raw navy beans appears to be due to (a) the unavailability of methionine, (b) impaired utilization of methionine, (c) deficiency of methionine, and (d) low food intake. The multiplicity of these "toxic" effects resulted in the death of rats fed raw beans. The presence of a toxic factor(s) other than trypsin inhibitor and hemagglutinins in the navy beans has been postulated.

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1964

ACKNOWLEDGMENTS

I owe a special debt of gratitude to Dr. R. J. Evans for his encouragement, assistance and criticisms throughout this investigation. His help during all the days of my present study can never be adequately acknowledged.

I should like to extend my sincere thanks to Dr. S. L. Bandemer and Dr. R. W. Leucke for their help and suggestions during the investigation. Thanks are also due to Dr. R. G. Hansen, Dr. P. J. Schiable and Dr. O. Mickelson for their interest in the present study.

Dr. K. K. Keahey and Dr. C. Whitehair were extremely helpful in providing the results on histopathological studies. The generous supply of navy beans provided by Dr. M. W. Adams is gratefully acknowledged.

I am deeply grateful to Michigan Bean Shippers Association for financing the research project and Sir Dorabji Tata Trust, Bombay, India, for the partial travel grant.

Cooperation extended by Mr. L. J. Klever, Betty Baltzer and other collegues in the laboratory is highly appreciated.

To Barbara Gibson my thanks for her efforts and time in typing and correcting this manuscript.

To My Family

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INTRODUCTION

It has been known since 1917 (1) that the navy bean is an inferior source of protein for rats. Everson and Heckert (2) reported that rats fed 10% protein as raw navy beans in an otherwise nutritionally complete diet lost weight and died during the experiment. These authors also reported that autoclaving the beans improved the nutritive value of raw navy beans. The present work was undertaken in an attempt to explain just how and why rats fed raw navy beans lost weight and died. Studies were also conducted to see the effect of dietary supplementation and various other treatments such as heating and soaking to improve the nutritive value of navy beans.

CHAPTER I

EFFECT OF HEAT TREATMENT

The beneficial and adverse effects of heat on legume proteins have been reviewed by Liener (3). The beneficial effect of heat on most legumes has been generally attributed to the destruction of antinutritional factors such as trypsin inhibitor and hemagglutinin. Bowman (4) and Rigas et al. (5) have shown the presence of a partially heat labile trypsin inhibitor and a hemagglutinin in navy beans. Evans and Butts (6) studied the effect of excessive heat treatment on the destruction and inactivation of amino acids of soybean protein. The purpose of the present experiment was to determine the effect of different heating periods on the nutritive value of navy beans in order to ascertain the optimum heat treatment to obtain the most beneficial effects.

Experimental

Navy beans of the Sanilac variety (protein content 24.00 per cent) were ground into fine flour. Protein content was calculated from the nitrogen value (Nx6.25). Nitrogen was determined by the micro-Kjeldahl procedure. The procedure was essentially as follows: To a digestion

flask a suitably weighed quantity of the sample +1.0 ml of 10% $CuSO_4$ soln. + 2.0 ml conc. H_2SO_4 were added and the flask and contents were digested on the electrical heater until all the white fumes were distilled off. Three drops of 30% H_2O_2 were added and the flask was further heated until the mixture in the flask was clear. The mixture was distilled by heating in Kjeldahl apparatus by the addition of a few drops of 40% NaOH slowly until the material in the digestion flask turned black or gray. NH_3 thus liberated was absorbed in a catch flask containing 20 ml of 2% boric acid + 5 drops of 1% bromocresol green in alcohol as an indicator. The distillation was continued until the volume of the catch flask was about 50 ml. The distillate was then titrated with 0.0142 N H_2SO_4 and the nitrogen content of the sample was calculated as follows: 1 ml of 0.0142 N H_2SO_4 = 0.198 mgs N_2.

Heated samples were prepared by autoclaving the finely ground beans in shallow pans at a thickness not exceeding 1.0 cm at 121° C for 5, 15, 30, 60, and 240 min., after the desired temperature was reached. After autoclaving the samples were placed before a fan and allowed to dry at room temperature.

The percentage composition of the basal diet was as follows: sucrose, 30; corn oil, 6; Hegsted salt mixture, 4; vitamin diet fortification mixture, 2. Raw or autoclaved navy beans or vitamin free casein was

incorporated into the basal diet to provide a level of 10% protein (N x 6.25). Cornstarch was added to make the total to 100.

Protein quality was evaluated using weanling albino rats of Hoppert's strain, 21 to 24 days old and weighing 45-55 g. Rats were divided into groups of 6 animals, each housed in an individual wire-bottom cage and each group was equalized as nearly as possible with respect to sex and weight. Food and water were supplied ad libitum. Rats were weighed weekly and accurate records were kept of food consumption over the 4 week experimental period. Protein efficiency ratio (PER) was calculated as change in body weight in gram per gram of protein consumed.

Trypsin inhibitor activity was determined by the casein digestion method of Kunitz (7) as follows: 5 g of navy bean flour was extracted with 50 ml of 1% NaCl solution. One ml of the original extract or a suitable dilution thereof was mixed with 1.0 ml containing 50 mcg trypsin (2x crystaline salt free trypsin) dissolved in phosphate buffer, pH 7.6. The reaction tubes were brought to 37° C in an incubator and the reaction was then started by addition of 1.0 ml of 1% casein in phosphate buffer, pH 7.6. Casein used was of "Hammarsten" quality. After 20 minutes the reaction was stopped by the addition of 3 ml of 5% TCA. After centrifugation the optical density

of the clear supernatant was read against a blank, prepared in the same way except that TCA was added before the addition of casein, at 280 mμ in a Beckman DU Spectrophotometer.

Along with trypsin inhibitor assay, the digestion of casein by trypsin was determined. The trypsin inhibitor activity in terms of trypsin inhibitor units (TIU) was expressed as the tryptic units inhibited, the tryptic unit being defined as the increase of one unit of optical density at 280 mμ per minute of digestion under the experimental conditions.

Determination of hemagglutinating activity was made according to the method of Liener (8). To 5 g of the bean sample in a 50 ml volumetric flask were added 1% NaCl, and the suspension was shaken vigorously.

A suspension of chicken red blood cells was collected by heart puncture in an oxalated tube and centrifuged. The sedimented cells were washed with saline at least three times, or until the supernatant fluid was clourless and diluted to give a final red blood cell concentration of 4% (V/V).

In performing the hemagglutininating test, decreasing volumes of the original navy bean extract were added to a series of tubes. To each tube containing 0.5 ml of diluted extract was added 0.2 ml of 4% suspension of the red blood cells. The tubes were incubated at 37° C for

one hour after which the agglutination was read by shaking the tube and noting the clumping of the cells. The cells which had not been agglutinated dispersed readily to form a uniform suspension. A tube containing 9.5 ml saline plus 9.2 ml of the cell suspension served as a negative control for this purpose. The agglutination test was positive when the cells were not suspended by shaking. Hemagglutinin unit (HU) was defined as the least amount of hemagglutinin which will produce positive evidence of agglutination under the experimental conditions.

Results and Discussion

The effect of various heating periods on the nutritive value of navy beans as measured by rat growth is given in Table 1. Rats fed raw navy beans as a source of protein lost weight and died within 20-26 days. On the other hand, rats fed navy beans autoclaved for 5 min. Gained weight, and there was a considerable increase in total food intake over that of rats fed raw beans. However, the lower PER values for rats fed beans autoclaved for longer than 5 minutes indicate the adverse effect of heat treatment on the nutritive value of navy beans. It is likely that these PER values may have been caused by the destruction or inactivation of essential amino acids in the manner described by Evans and Butts (6).

TABLE 1.--Effect of feeding raw and heated beans on the growth of rats.

Protein Source	Average Change in wt.	Average Food Intake	Protein Efficiency Ratio (PER)
	g	g	
Casein	98.0	286	3.41 ± 0.11
Raw Beans	-13.2	92	100% mortality observed[a]
Beans autoclaved for 5 min.	35.0	226	1.57 ± 0.10
Bean autoclaved for 15 min.	25.0	197	1.26 ± 0.12
Beans autoclaved for 30 min.	21.0	198	1.09 ± 0.06
Beans autoclaved for 1 hour	12.0	167	0.67 ± 0.11
Beans autoclaved for 4 hours	- 8.0	164	-0.48 ± 0.09

[a]Average change in weight and average food intake were calculated over 3 week experimental period whenever the mortality of rats was observed here or hereafter

The effect of heat treatment on trypsin inhibitor and hemagglutinin activities was investigated (Table 2) because these have been suggested as the growth inhibiting materials in navy beans (3). Autoclaving of raw beans for 5 minutes destroyed 80% of the trypsin inhibitor activity and 100% of the hemagglutinin activity. Thus the low nutritive value of raw navy beans may be due to the presence of heat labile trypsin inhibitor and hemagglutinin. Honavar et al. (9) reported growth inhibition of rats fed purified hemagglutinin isolated from beans (Phaseolus valgaris).

Borchers (10) fed various dietary levels of raw soybean meal to weanling rats to see if there is a "toxic" factor in raw soybeans. Table 3-I shows the effect on rat growth of substituting various levels of raw navy beans for casein in a 10% protein diet. Rat growth and total food intake decreased as the level of beans in the diet increased. Rats fed raw navy beans at a 20% protein level died within 5-10 days from the beginning of the experiment which is quite consistent with the hypothesis that the effect of a toxic factor should be enhanced at a higher level of intake. Navy beans thus appear to contain a "toxic" factor(s).

Table 3-II shows the effect on rat growth of replacing casein with autoclaved beans. PER values obtained with diets containing beans at 2.5, 5.0, or 7.5% protein level

TABLE 2.--Effect of heat on hemagglutinating and trypsin inhibitor activities of crude extracts of beans.

Heat Treatment	Hemagglutinating Activity HU/ml.	Trypsin inhibitor Activity TIU x 10^{-3}/ml.
None	512	2250
5 min., 121°C	0	400
15 min., 121°C	0	260
30 min., 121°C	0	260
60 min., 121°C	0	0
240 min., 121°C	0	0

TABLE 3.--Effect of feeding varying levels of casein and beans on the growth of rats.

Source of Protein Casein	Protein Beans	Average Change in Wt.	Average Food Intake	Protein Efficiency Ratio (PER)
(% in diet)		g	g	
Part-I (Raw Beans)				
10.0	0.0	82.0	308	2.67 ± .08
7.5	2.5	44.0	233	1.89 ± .15
5.0	5.0	2.0	150	0.16 ± .06
2.5	7.5	- 8.0	153	-0.71 ± .13
0.0	10.0	-12.3	84	100% mortality observed
0.0	20.0	100% mortality in 5-10 days.		
Part-II (Autoclaved Beans)				
10.0	0.0	85.0	305	2.85 ± 0.13
7.5	2.5	81.0	289	2.81[a] ± 0.07
5.0	5.0	75.0	280	2.67[a] ± 0.12
2.5	7.5	64.0	266	2.37[a] ± 0.17
0.0	10.0	47.0	255	1.84[b] ± 0.07
0.0	20.0	90.0	260	1.62[b] ± 0.06

[a] t value is not significant

[b] t value is significant

When compared to casein group at 5 per cent significance level.

were not significantly different from that of casein ($P > 0.05$) but the PER value obtained with 10% bean protein was significantly less than that of casein ($P < 0.05$). This observation may have some practical implications in certain countries where there is a shortage of animal protein food. It is interesting to note that rats fed autoclaved beans at a 20% protein level grew as well as those fed casein at a 10% protein level. It was found that when the bean protein level was increased from 10% to 20% the methionine-cystine content of the ration was increased from 0.2 to 0.40 compared to 0.38% in the 10% casein diet. The poor growth of rats fed autoclaved beans at a 10% protein level could be due to a deficiency of sulfur containing amino acids.

Summary

An attempt was made to explain the beneficial and adverse effects of heat treatment on the nutritive value of navy beans. Rats fed raw navy beans as a sole source of protein lost weight and died within the experimental period. The results of feeding beans heated for various periods on the growth of rats indicated that heating for 5 minutes at 121°C was optimal. As a result of this finding the subsequent studies were made on the beans autoclaved at 121°C for 5 minutes. Autoclaving the beans

also destroyed the trypsin inhibitor and hemagglutinin. The low nutritive value of raw navy beans appears to be due to the presence of a heat labile toxic factor(s) and the deficiency of sulfur amino acids.

CHAPTER II

AMINO ACID SUPPLEMENTATION

The beneficial effect of amino acid supplementation on the nutritive value of legume proteins is well known and is generally attributed to a deficiency of amino acids in them (11, 12). Bandemer and Evans (13) compared the amino acid composition of navy beans to the FAO protein reference pattern (14) and reported that methionine is the most limiting amino acid. Recent reports of Borchers (15) and of Booth et al. (16) indicate that amino acid supplementation improved the growth of rats fed raw soybean meal and in this connection Borchers (15) suggested that a growth inhibitor present in raw soybean meal increased the dietary requirement for amino acids. The previous results as shown in Table 3-I might also be explained on the basis of an intensification of an amino acid imbalance, which, if true, could be corrected by amino acid supplementation. For example, a calculation of the actual intake of raw beans for the diets containing 2.5, 5.0, 7.5 and 10% of bean protein shows that rats actually consumed 23, 30, 40, and 32 g. of raw beans respectively. Thus there was not significant difference in the navy bean

intake between the groups. It would therefore seem that the inbalance may also have been a possible cause of low nutritive value of the navy beans.

In the light of above considerations the present work was undertaken to see if the observed growth inhibition of rats fed raw navy beans is a result of amino acid deficiency or amino acid imbalance.

Experimental

Preparation of diets and the details of rat feeding experiment were described in a previous chapter.

Amino acids were determined by microbiological assay procedures as follows: Amino acids analyses for all amino acids except tryptophan were made on acid hydrolysates of the samples. Alkaline hydrolysis was used for the estimation of tryptophan. Acid hydrolyses were carried out by autoclaving one gram navy bean sample with 25 ml of 3.0 N HCl (5.0N NaOH for alkaline hydrolysis) for 9 hours. The hydrolysates were cooled, 2 ml of 2.5 M sodium acetate solution added, pH adjusted to 6.8, and made up to a suitable volume and filtered. The hydrolysates were assayed at 3 different concentration levels. Lactobacillus arabinosus 17/5 was used in assaying leucine, isoleucine, valine and tryptophan while Luconostoc mesenteroides P-60 was used in assaying lysine, phenylalanine, histidine and methionine. Arginine and threonine were determined by assay with Streptococcus faecalis.

The preparation of the media and the details of the assay procedure were followed as recommended by Barton-Wright (17). The procedure was essentially as follows: One ml aliquot of the double-strength basal medium was added to each test tube by means of burette. A suitable aliquot of the standard amino acid solution or sample extract was then added. The contents of each test tube were diluted to a total volume of 2 ml, using distilled water. For establishing a standard curve, the range of the amino acid concentration was one-fifth of that recommended by Barton-Wright (17).

After the volume of the tubes had been finally adjusted to 2 ml they were capped with aluminum thimbles and sterilized in an autoclave at 10 lb. pressure for 10 min. The tubes were allowed to cool and were then inoculated. The inoculum was prepared by inoculating an enriched medium in a sterilized tube with a portion of a stab culture by means of a sterile platinum needle. After 18-20 hours of the incubation period the contents of the tube were centrifuged aseptically, and the cells were washed with sterile saline solution at least two times. The cells were then suspended in 10 ml of saline solution and 1.0 ml of this suspension was added to about 20 ml sterile saline solution. For inoculation of the tubes, one drop of this diluted suspension was added by means of sterile pipette to each tube.

The tubes were incubated at 37°C for 68-72 hour. At the end of the incubation period, the lactic acid produced in the tubes was titrated against 0.02 N NaOH (0.01 N NaOH was used in the case of S. faecalis assay) using bromothymolblue as an indicator.

The amino acid value was determined by taking the mean of direct readings from the standard curve at the three concentration levels provided the values thus obtained did not differ among themselves by more than ±10 per cent.

Results and Discussion

Comparison of the amino acid composition of raw or autoclaved navy beans with that of the FAO reference pattern (14) shows bean protein to be deficient primarily in methionine. However, as indicated in Table 4 a comparison with the essential amino acid pattern proposed by Rose for growing rats (18) and with the amino acid composition of casein show that navy beans are deficient in most of essential amino acids. The effect of supplementing navy beans with amino acids are presented in Table 5. Supplementation of the raw bean diet with methionine or with all deficient amino acids did not overcome the growth depression although the mortality was reduced. However, rats fed autoclaved bean diet supplemented with methionine along (group 5) or with

TABLE 4.--Essential amino acid composition of casein, of raw and autoclaved beans, of the FAO reference pattern, and of the pattern proposed by W. C. Rose for growing rats (expressed as g amino acid/100 g of protein)

Amino Acids	Casein[a]	Raw Beans	Auto-claved Beans	FAO Pattern	Rose Pattern
Arginine	4.3	5.6	5.5	-	2.0
Histidine	3.1	2.6	2.8	-	4.0
Isoleucine	6.5	4.6	5.4	4.2	5.0
Leucine	9.7	7.5	8.4	4.8	8.0
Lysine	8.7	7.1	7.2	4.2	10.0
Methionine	3.0	1.0	1.0	4.2[b]	4.0
Phenylalanine	5.8	5.1	5.0	2.8	7.0
Threonine	4.7	4.6	5.3	2.6	5.0
Tryptophan	1.5	1.4	1.8	1.4	2.0
Valine	7.4	4.8	5.6	4.2	7.0

[a]Data obtained from Nutritional Biochemicals Corporation Cleveland, Ohio.

[b](total sulfur-containing).

TABLE 5.--Growth of rats fed rations containing raw and autoclaved beans supplemented with essential amino acids.

Group No.	Protein source	Average Change in wt.	Average Food intake	Protein Efficiency Ratio (PER)
		g	g	
1	Casein	106.0	352	3.18
2	Raw Beans	-14.6	96	100% mortality
3	Autoclaved beans	42.0	221	1.90
4	Raw Beans plus methionine[a]	-15.0	102	50% mortality
5	Autoclaved beans plus methionine[a]	94.0	300	3.13
6	Raw beans plus Amino Acids[b]	-16.0	103	-1.48
7	Autoclaved beans plus amino acids[b]	102.0	320	3.19
8	Raw beans plus amino acids[c]	-16.3	110	-1.48
9	Autoclaved beans plus amino acids[c]	100.0	312	3.20

[a]Methionine added to bring to level recommended by FAO.

[b]Amino acids added to bring to level proposed by Rose.

[c]Amino acids added to bring to level furnished by casein.

all deficient amino acids grew as well as rats fed casein diet. There appears to be no advantage in adding any amino acid other than methionine to the autoclaved bean diet. The results indicate that the FAO reference pattern supplies sufficient amino acids for growth of rats. They also indicate that the growth inhibition of rats fed raw navy beans does not arise from an amino acid imbalance or deficiency in the diet.

Summary

The effect of feeding raw and autoclaved navy beans supplemented with deficient essential amino acids on the growth of rats was investigated. Raw navy beans did not support growth of rats even when supplemented with all of the deficient amino acids. Rats fed autoclaved beans supplemented with methionine alone or with all deficient amino acids grew as well as those fed the casein diet. It is concluded that amino acid deficiency or imbalance is not a cause for the low nutritive value of raw navy beans.

CHAPTER III

METHIONINE, VITAMIN B_{12}, AND ANTIBIOTICS SUPPLEMENTATION

The effectiveness of methionine, vitamin B_{12} and antibiotics supplementation in improving the nutritive value of soybean protein has been reported by a number of investigators (19, 20, 21, 22, 23). Borchers (24) observed an apparent increased requirement for methionine in rats fed raw soybean meal. Barnes et al. (25) suggested that the growth inhibitor in raw soybean specifically interferes with tissue utilization of methionine. This would explain why supplementary methionine is necessary for the proper utilization of soybean protein. The role of vitamin B_{12} may be attributed to its methionine sparing action as suggested by Liener and Schultze (26). However, the mode of action of antibiotics in overcoming the growth depression of rats fed raw soybean meal is not known. It is the purpose of the present chapter to determine the effectiveness of methionine, vitamin B_{12} and antibiotics supplementation in overcoming growth inhibition of rats fed raw navy beans.

Experimental

Preparation of diet and the details of rat feeding experiment were described in detail before.

All supplements were added at the expense of starch at the following levels:

Methionine: 0.6% DL methionine

Vitamin B_{12}: 50 mg of 0.1% triturated vitamin B_{12} per kilo of diet.

Antibiotics: 0.1% procaine penicillin and 0.1% streptomycin sulfate.

Results and Discussion

Table 6 shows the effect of supplementing raw beans with methionine, vitamin B_{12}, or antibiotics on the growth of rats. Rats fed raw beans lost weight and died within 20-26 days. Supplementation with methionine and/or vitamin B_{12} failed to correct the growth depression, but did reduce mortality. Supplementation with antibiotics prevented the growth depression and mortality and when fed in combinations with methionine and vitamin B_{12} promoted limited growth. In all these experiments the food intake was considerably reduced.

Results of feeding autoclaved beans supplemented with methionine, vitamin B_{12} and antibiotics are presented in Table 7. Rats fed autoclaved beans gained weight although not at a rate comparable to those fed casein.

TABLE 6.--Effect of supplementing raw beans with methionine, vitamin B_{12} and antibiotics on the growth of rats.

Group No.	Protein source	Average Change in wt.	Average Food intake	Protein Efficiency Ratio (PER)
		g	g	
1	Raw beans	-14.4	98	100% mortality
2	Raw beans plus methionine	-14.8	102	50% mortality
3	Raw beans plus vitamin B_{12}	-13.0	116	33% mortality
4	Raw beans plus methionine plus vitamin B_{12}	- 6.5	135	-0.53 $\pm$ 0.17
5	Raw beans plus antibiotics	0.3	129	0.03 $\pm$ 0.10
6	Raw beans plus vitamin B_{12} plus antibiotics	1.0	124	0.09 $\pm$ 0.10
7	Raw beans plus methionine plus antibiotics	5.2	130	0.44 $\pm$ 0.14
8	Raw beans plus methionine plus vitamin B_{12} plus antibiotics	14.0	131	1.07 $\pm$ 0.13

TABLE 7.--Effect of supplementing autoclaved beans with methionine, vitamin B_{12} and antibiotics on the growth of rats.

Group No.	Protein source	Average Change in wt.	Average Food intake	Protein Efficiency Ratio (PER)
		g	g	
1	Casein	127.0	360	3.52 ± 0.07
2	Autoclaved beans	60.0	320	1.88 ± 0.04
3	Autoclaved beans plus methionine	120.0	350	3.43 ± 0.07
4	Autoclaved beans plus vitamin B_{12}	68.0	330	2.06 ± 0.08
5	Autoclaved beans plus antibiotics	71.0	330	2.13 ± 0.08
6	Autoclaved beans plus vitamin B_{12} plus antibiotics	74.0	320	2.32 ± 0.08
7	Autoclaved beans plus methionine plus vitamin B_{12}	121.0	354	3.39 ± 0.18
8	Autoclaved beans plus methionine plus antibiotics	136.0	360	3.78 ± 0.13
9	Autoclaved beans plus methionine plus vitamin B_{12} plus antibiotics	134.0	360	3.79 ± 0.13

Rats fed the autoclaved bean diet supplemented with methionine grew as well as those fed casein. The PER value obtained with autoclaved beans supplemented with vitamin B_{12} was not significantly different from that with autoclaved beans alone; however, the PER values for autoclaved beans supplemented with antibiotics were significantly different from that of autoclaved beans or autoclaved beans supplemented with methionine. There was no significant difference between the PER values for autoclaved beans plus methionine and autoclaved beans plus methionine, vitamin B_{12} and/or antibiotics. It is unlikely, therefore, that vitamin B_{12} or antibiotics was sparing for methionine in this investigation. The slight beneficial effect of antibiotics supplementation on PER over that of unsupplemented autoclaved bean diet might be caused by an effect on digestibility or absorption of the nutrients as a result of its action on intestinal micro-flora (23) or on intestinal cell walls (27).

In the light of above results an explanation for the beneficial effect of antibiotic supplementation in overcoming the growth depression of rats fed raw beans remains to be determined. Several investigators have reported the presence of hemagglutinin in navy beans (28, 5). Honavar et al. (9) observed growth inhibition of rats fed a purified hemagglutinin fraction isolated from Phaseolus vulgaris. It has been suggested (29) that the

action of hemagglutinin might be to combine with the intestinal cell wall thus interfering with absorption of nutrients. If this is the case the role of antibiotics may be action on the intestinal cell linings, thereby increasing the absorption of nutrients. However, the above explanation does not hold true for soybean meal as no significant difference in digestibility was observed between raw and heated soybean meal (30,31). Therefore, other possibilities still exist for explaining the mode of action of antibiotics. Borchers (32) reported that the soybean growth inhibitor exists in a "bound" form and suggested the possibility that an enzyme or microorganisms associated with the raw soybean meal liberates the "bound" form of inhibitor. It is possible that the role of antibiotics is to inhibit the enzyme responsible for the liberation of "bound" growth inhibitor. The inhibition of enzyme systems by antibiotics is not uncommon in the literature. Hartsook et al. (33) reported the inhibition of kidney xanthine dehydrogenase in the chick and suggested that antibiotics cause increased nitrogen retention.

Another explanation for the supplementary effect of antibiotics would be that they inhibit the growth of certain intestional microorganisms which might act on the raw beans releasing "toxic" or growth inhibiting substance.

Summary

Supplementation of raw beans with methionine and/or Vit. B_{12} promoted limited growth. Rats fed autoclaved beans supplemented with methionine grew as well as rats fed casein. Protein efficiency ration (PER) values of autoclaved beans supplemented with Vit. B_{12} and/or antibiotics were significantly lower than those of beans supplemented with methionine. Antiobiotics may act to overcome growth depression by increasing the digestibility or absorption of nutrients and it is suggested that antibiotics may also act by inhibiting an enzyme involved in liberating a "bound" growth inhibitor from raw beans.

CHAPTER IV

HISTO-PATHOLOGICAL STUDIES

The gross changes observed in rats fed raw navy beans were (a) growth inhibition; (b) reduced food consumption; (c) excessive flow of urine; (d) diarrhea and ultimately (e) death. Booth et al. (16) studied the morphological changes of heart, lungs, thyroid, testes, spleen, liver, pancreas, kidney, adrenals, and intestine and found that all organs of the animals fed raw soybean meal were normal except the pancreas which was hypertrophic. These authors suggested that the growth inhibition of rats fed raw soybean meal may be due to direct stimulation of the pancreas resulting in excessive flow of critical amino acids contained in the pancreatic enzymes which are then excreated in the feces. However, studies made by Saxena et al.(34) indicate that the growth depression in chicks fed raw soybean meal may be due to inability of the hypertropic pancreas to supply the necessary digestive enzymes. The present investigation was undertaken in an attempt to elucidate the mechanism of growth inhibition of rats fed raw navy beans, from the standpoint of the morphological changes which occur.

Experimental

Preparation of diet and the details of rat feeding experiments were described in detail previously. Rats were fed the experimental diets for 20 days and casein was used in a control diet. At the end of the experimental period the rats were euthanitized and the selected organs were excised, blotted and weighted. Sections of tissues were fixed in 10% neutral formalin. Representative samples of each tissue were embedded in paraffin, sectioned at 6 microns and stained with hematoxylin and eosin. Special stains were used where needed.

Results and Discussion

Table 8 shows the effect of feeding experimental diets on the growth of rats. Rats fed raw bean diet lost weight and consumed considerably less food compared to those fed the autoclaved bean diet. Although protein efficiency ratio value for autoclaved bean diet is greatly increased over that of raw bean diet, it is significantly less than that of the animals fed the casein diet. This may be due to a methionine deficiency in the navy bean diet.

Rats fed casein or autoclaved bean diet appeared to be normal. However rats on raw navy bean diet were emaciated, debilitated, had rough hair coats and were wet around the genitalia. There was paleness of the mucous

TABLE 8.--Effect of feeding experimental diets on the growth of rats.

Protein source	Average Change in weight	Average food intake	Protein Efficiency Ratio (PER)
	g	g	
Casein	75.6	232	3.28 ± 0.12
Raw navy beans	-12.8	84	-1.54 ± 0.10
Autoclaved navy beans	25.4	166	1.58 ± 0.11

membranes and the liver was yellowish in color. The abdominal cavity contained excess amounts of serous fluid.

The effect of feeding experimental diets on the weight of different organs of the rats is shown in Table 9. The data indicate that the heart and kidneys of rats fed raw navy beans are significantly increased ($P<0.05$) in weight when compared to the weight of the organs of animals fed casein diet. Phadke and Sohonie (35) also observed the similar differences in the weight of these organs of rats fed field bean diet. In the present investigation it is likely that the unavailability of amino acids might be the cause of the differences in the weight of organs of animals fed raw navy bean diet as there was

TABLE 9.--Effect of feeding experimental diets on the weight of different organs of rats. (Average weight of organs expressed in g/100 g of body weight).

Organ	Diet		
	Casein	Raw Navy Beans	Autoclaved Navy Beans
Liver	4.20 ± 0.06	3.84[b] ± 0.20	4.94 ± 0.20
Heart	0.50 ± 0.001	0.63[a] ± 0.001	0.55[b] ± 0.002
Spleen	0.27 ± 0.04	0.18[b] ± 0.04	0.28 ± 0.04
Kidney	0.91 ± 0.02	1.40[a] ± 0.06	1.06[b] ± 0.17
Adrenals	0.031 ± 0.005	0.032[b] ± 0.005	0.023 ± 0.001

[a]t value is significant at 5% level when compared to casein group.

[b]t value is not significant at 5% level when compared to casein group.

no significant differences in the weight of different organs of animals fed autoclaved bean diet as compared to those on casein diet.

The following tissues were examined microscopically: kidney, liver, adrenal gland, stomach, duodenum, jejunum, ileum, pancreas, bone, skin, esophagus, trachea, and lymph node. The morphological changes observed in rats fed raw navy bean diet were as follows:

Liver.--(Figure 1) Increased numbers of vacuoles were present in hepatic cells and these were prominent near the periphery but were distributed throughout the lobule. There was some derangement of liver cords and confluence of cytoplasm of adjacent cells. The nuclei were more densely packed indicating a relative decrease in size and the nuclei contained enlarged nucleoli. Fatty metamorphosis was marked in sections stained with oil red O. The similar findings were noted in rats fed diet deficient in essential amino acids (36, 37).

Pancreas.--(Figure 2) According to micrometer measurements acinar cells were reduced in size. The cytoplasm was almost devoid of secretory granules, occupied less area and uniformly stained deeply basophilic. Acinar arrangement and cell outline were indistinct.

Thyroid.--Most of the follicles were small and contained little or no colloid, therefore rendering the thyroid gland more cellular per unit area.

Kidney.--A few distal convulated tubules and most of Henle's loops were dilated with the lining epithelium pressed against the basement membrane in a thin layer.

Esophagus.--A wide layer of keratin was present on the epithelial surface and this partially filled the esophagal lumen. Foci of cellular remnants suggestive of active desquamation were present.

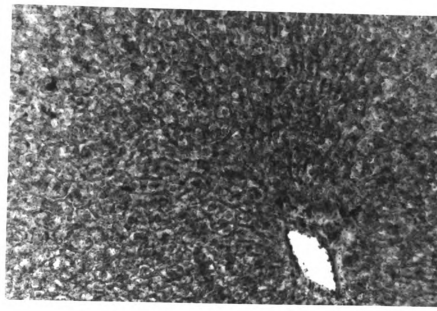

Fig. 1A.--Liver from a control rat fed a casein diet. Oil Red O. x187.

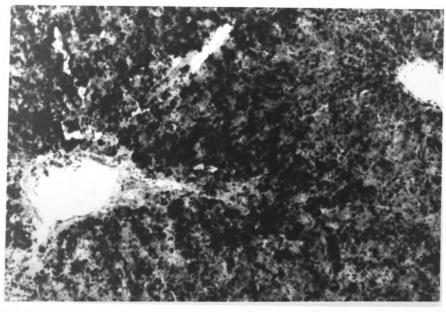

Fig. 1B.--Liver from a rat fed a raw navy bean diet. Note fatty metamorphosis. Oil Red O. x187.

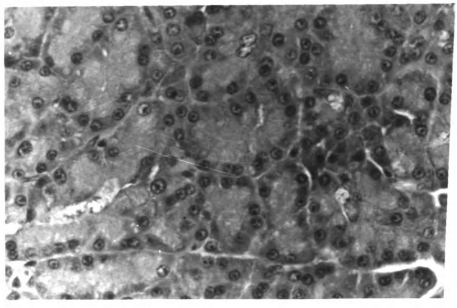

Fig. 2A.--Pancreas from a control rat fed a casein diet. H. and E. x469.

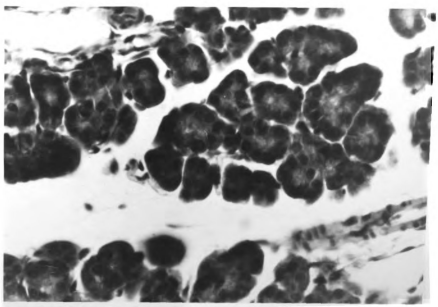

Fig. 2B.--Pancreas from a rat fed a raw navy bean diet. Note atrophic acini. H. and E. x469.

Skin.--There was mild hyperkeratosis with excess keratin attached to the free surface of the skin.

The morphological changes observed in rats fed autoclaved bean diet could be due to the methionine deficiency in bean diet because no microscopic lesions were found in the tissues of rats fed autoclaved bean diet supplemented with methionine.

It appears, then, that the morphological changes observed in the tissues of rats fed raw navy beans are mainly due to the deficiency and unavailability of critical amino acids such as methionine. Bowman (4) has shown the presence of heat labile trypsin inhibitor in navy beans, the action of which would be expected to accentuate the deficiency of critical amino acids by inhibiting the action of trypsin. It is likely that the beneficial effect of autoclaving the navy beans in inhibiting the morphological changes in rats could be due to the destruction of trypsin inhibitor and thereby increasing the availability of critical amino acids.

The possibility exists that there may be present a factor in raw navy beans which inhibits the production by the pancreas of the necessary digestive enzymes as postulated by Saxena et al. (34) in the case of raw soybeans. It may also be that a factor stimulates the production of pancreatic enzymes resulting in the loss of critical amino acids as proposed by Booth et al. (16) and Haines

and Lyman (38) in explaining the low nutritive value of raw soybeans. However, morphological changes observed in the pancreas of animals fed raw navy beans did not allow to differentiate between these two mechanisms. Because the condition in which the cytoplasm of the pancreas was devoid of secretory granules could arise as a result of either mechanism described above. Recently Barnes et al. (25) proposed a hypothesis to explain the mechanism of growth inhibition of rats fed raw soybeans. These authors concluded that a growth inhibitor of soybeans specifically interferes in the tissue utilization of methionine by the tissues. It is likely that a factor present in navy beans also specifically interferes in the normal metabolism of methionine by impairing either the availability of methionine to the tissues or the utilization of methionine by the tissues.

In the light of the above discussion it can be concluded that the growth inhibition of rats fed raw navy beans could be partly due to the impairment in the availability of critical amino acids and partly due to the methionine deficiency. Moreover, this condition can further be aggravated by the low food intake resulting in the limited supply of amino acids in the animals fed raw navy beans.

Summary

The experimental diet of raw navy beans produced the most severe changes which included; growth inhibition, reduced food consumption, pancreatic acinar atrophy, fatty metamorphosis of the liver, thyroid follicular atrophy and hyperkerotosis of the esophagus and skin.

Significant morphological changes were not observed in the organs of rats fed autoclaved navy bean diet as compared to the control group.

CHAPTER V

DIGESTIBILITY STUDIES

Part I

Considerable progress has been made in the application of *in vitro* chemical and microbiological methods to the study of factors influencing digestibility. Melnick *et al.* (39) have stressed the importance of rate of enzymatic hydrolysis of dietary protein in determining the availability of amino acids. Evans *et al.*(40) have applied both *in vitro* enzyme digestion tests and *in vivo* tests with growing chickens to the problem of determining the effects of heat treatment on protein quality of soybean meals. They observed positive correlation between protein efficiency for the chick and the susceptibility of the sample protein to *in vitro* enzymatic hydroysis. The present investigation was undertaken to study the rate of release of amino acids of navy bean protein by certain digestive enzymes.

Experimental

The study was carried out on raw beans and the beans heated at 121° for 5 minutes in the autoclave.

Enzymatic digestion: Peptic digestion was carried out on 8.0 g. samples of raw or heated beans suspended in

100 ml. of 0.1 N HCl. The pH was adjusted to 1.8, and 50 mg. of pepsin (1:10,000) was added. The resulting mixture was incubated for 24 hours at 37° C.

Tryptic digestion: 8.0 g samples of raw or heated beans were transferred to different flasks, and each sample was suspended in 100 ml. of 0.1 M Sorenson's phosphate buffer, pH 7.6, and to this was added 50 mg of trypsin (1:300). The flasks were incubated for 24 hours at 37° C.

For peptic-tryptic digestion the pepsin digest was subsequently adjusted to pH 7.6, and 50 mg. of trypsin was added. The resulting misture was incubated for another 24 hours. The reaction mixture in each flask was covered with toluene in order to avoid a microbial contamination.

After the desired period of incubation the enzyme activity was stopped by heating the flasks for 30 minutes on a steam bath. The insoluble residue was removed by centrifugation. Amino acid analyses were made on enzymatic hydrolysates of raw and heated beans. Microbiological assay was used to determine amino acids. The procedure was described before.

Results and Discussion

The relative amounts of amino acids liberated by digestive enzymes from raw and heated beans are given in Table 10. The values obtained for amino acids after trypsin or pepsin-trypsin digestion show that the rate of liberation of amino acids was considerably increased by autoclaving the

TABLE 10.--Liberation of amino acids from raw and autoclaved beans by pepsin, trypsin and pepsin-trypsin digestion as determined by microbiological assay[a] (expressed as percentage of amino acids in acid hydrolysates)

Amino Acids	Pepsin Digestion		Trypsin Digestion		Pepsin-Trypsin Digestion	
	Raw Beans	Autoclaved Beans	Raw Beans	Autoclaved Beans	Raw Beans	Autoclaved Beans
Arginine	45	34	13	24	63	23
Histidine	6	4	30	40	22	47
Isoleucine	46	36	8	27	33	54
Leucine	50	40	7	20	14	36
Lysine	4	3	30	63	24	66
Methionine	18	18	31	43	40	56
Phenylalanine	17	17	16	52	36	64
Threonine	76	42	7	31	37	33
Tryptophan	43	25	21	32	50	45
Valine	41	35	6	36	41	45

[a]No correction for enzyme is made in the assay.

beans. Values for pepsin digestion show a very different pattern of liberated amino acids. This may be because of the growth promoting properties of certain peptides (41, 42, 43) or the ability of the assay organism to utilize these peptides (44). In view of these findings it would appear that in vitro digestion studies by pepsin alone is not reliable in assessing the nutritive value of protein foodstuffs, a conclusion which has also been reached by DeBaum and Connors (45). Bowman (4) has shown the presence of trypsin inhibitor in navy beans which inhibits the in vitro digestion of casein by trypsin and which is also partially heat labile. The improvement in digestibility of navy beans by autoclaving for 5 minutes may be attributed to the destruction of such inhibitors.

Summary

In vitro digestion studies showed that autoclaved beans were more digestible than the raw beans as revealed by the liberation of essential amino acids.

Pepsin did not show any effect on the digestibility of autoclaved beans. However, trypsin and pepsin-trypsin digestion studies indicate the beneficial effect of autoclaving on the digestibility of navy beans.

Part II

The observation that antibiotics in combination with methionine and vitamin B_{12} promoted the growth of rats fed

raw navy beans suggested that a growth inhibitor of navy beans specifically interfers in the availability and/or utilization of methionine. Previous finding has established that raw navy beans were poorly digested *in vitro* by certain digestive enzymes. This prompted the hypothesis that the availability of methionine may be a major cause for the growth inhibition of rats fed raw navy beans. Therefore, the present investigation was undertaken to study the digestibility in the young rats and to examine the possible role of antibiotics in the digestibility of navy bean protein.

Experimental

The composition of the basal diet employed is described before.

Male young albino rats 26-28 days old, weighting 65-75 g were used in all studies. The animals were divided into groups of 8 rats each housed in an individual wire-bottom metabolic cage, and each group was equalized as nearly as possible with respect to initial weight. Food and water were supplied *ad libitum*. The animals were fed the diet under study for a seven-day period. Feces were collected for the last 4 days of each period and dried at 100°C. Food consumption was determined and the animals were weighed at the end of the experiment. The following procedure was adopted to keep the daily record of food intake. To 20 g of the diet was added 10 ml of water and the mixture was stirred well.

Food intake was calculated on dry-weight basis (wet-weight x 2/3) neglecting the loss of water by evaporation after being allowed to stand overnight in cage.

Nitrogen content was determined by a micro-Kjeldahl procedure and methionine by microbiological assay as recommended by Barton-Wright (17) using Leuconostoc mesenteroids as described before.

Nitrogen or methionine digestibility in percentage was calculated by the following formula:

$$100 \times \frac{\text{'N' (or methionine) intake - 'N' (or methionine) excreted}}{\text{'N' (or methionine) intake}}$$

Antibiotics were added in the diet at the expense of starch at the following level: 0.1% procaine penicillin + 0.1% streptomycin sulfate.

Results and Discussion

The results presented in Table 11 indicate that nitrogen was poorly absorbed in the animals fed raw navy bean diet as compared to those fed autoclaved bean diet. Although antibiotics increased the absorption of nitrogen in the rats receiving raw bean diet, it is significantly less than that for the rats receiving autoclaved bean diet. No significant difference was observed in the nitrogen digestibility by rats fed autoclaved bean diet and autoclaved bean diet with added antibiotics.

Apparant digestibility data for methionine (Table 11) indicate that more methionine was excreted through feces than

TABLE 11.--Digestibility of nitrogen and methionine by rats fed navy beans.

Protein source	Average weight change g/day	Average food intake g/day	Protein Efficiency Ratio (PER)	Apparent digestibility[a]	
				Nitrogen %	Methionine %
Raw Beans	-1.37	6.24	-2.10	41.80±3.62	-7.1±5.40
Autoclaved beans	+1.75	11.80	+1.48	80.35±0.37	59.5±1.62
Raw beans antibiotics	-0.60	5.74	-1.04	56.22[b,c,d]± 2.00	44.6[b,c,d]± 2.00
Autoclaved beans plus antibiotics	+1.30	8.26	+1.57	82.80±1.21	86.8±1.72

[a] Not corrected for endogneous excretion
[b] [c] [d] Highly significant (P<0.01) compared to appropriate treatment.
[c] Highly significant (P<0.01) compared to raw beans
[d] Highly significant (P<0.01) compared to autoclaved beans.

was ingested by the rats fed raw navy bean diet. Percentage digestibility was greatly increased in the animals fed autoclaved bean diet. The addition of antibiotics also increased the availability of methionine. This was true whether raw or autoclaved navy beans were used in the diet.

It appears then that the supplementary effect of antibiotics was in major part due to the improvement in the availability of nitrogen and especially of methionine in the animals fed raw navy bean diet. Bowman (4) has shown the presence of partially heat-labile trypsin inhibitor in navy bean the action of which is to be expected to accentuate the deficiency of methionine as a result of incomplete protein digestion. The lower digestibility of nitrogen and methionine observed in the present study may be due to the action of trypsin inhibitor resulting in the incomplete digestion of navy bean protein.

It is also likely that the increased loss of nitrogen and/or methionine in the feces of rats fed raw navy bean diet may represent endogeneous losses of amino acids through feces possibly due to the stimulation of pancreas as proposed by Haines and Lyman (38) and Booth et al. (16) in explaining the low nutritive value of soybeans. It may be that antibiotics somehow inhibit this pancreatic stimulation, a possibility which has been also discussed by Linerode et al. (46).

From the results presented in this investigation it can be concluded that availability of methionine is a major cause for the growth depression in rats fed raw navy beans and antibiotics somehow increase the availability of methionine to the animals. It can be suggested that a growth inhibitor of navy beans binds methionine to form an inhibitor-methionine complex, so that it is unavailable to the animals for the tissue protein synthesis. It is possible that antibiotics may hydrolyse the inhibitor-methionine complex thus making methionine available to the rats. This possibility involving the hydrolysis of a linkage has been discussed by Braham et al. (23) in explaining the supplementary effect of antibiotics on the nutritive value of raw soybeans. Another possibility is that antibiotics may combine with a growth inhibitor to form an inhibitor-antibiotics complex thus preventing the growth inhibitor from exerting its deleterious effect. It may also be that antibiotics act by inhibiting an enzyme involved in liberating a bound growth inhibitor from raw beans as suggested previously.

The beneficial effect of antibiotics supplementation on the digestibility of methionine by rats fed autoclaved bean diet as compared to that of unsupplemented autoclaved bean diet can be attributed to the case in absorption of the methionine or methionine peptides (23) through the thinning of the intestional cell walls as a result of antibiotics action (27).

The very observation that rats fed raw navy beans supplemented with methionine, vitamin B_{12} and antibiotics did not grow as well as rats fed autoclaved bean diet or autoclaved bean diet with proper supplementation suggests that the impairment in the methionine utilization may also be another defect in rats receiving raw bean diet.

Summary

The beneficial effect of supplementary antibiotics on the digestibility of nitrogen and methionine by the rats receiving raw navy bean diet has been observed. It is suggested that decreased absorption of nitrogen and methionine may be due to incomplete digestion of navy bean protein or may represent endogeneous losses of amino acids. It is concluded that the availability of methionine is a major factor in the low nutritive value of navy beans. Mechanism of action of antibiotics in improving the digestibility of bean protein is discussed.

CHAPTER VI

STUDIES ON TRYPSIN INHIBITOR AND HEMAGGLUTININS AS POSSIBLE GROWTH INHIBITORS

Part I

The beneficial effect of supplementary trypsin on the growth of rats (47) and on chicks (48) fed soybeans can be attributed to the trypsin and trypsin inhibitor complex formation as described by Kunitz (49), thereby neutralizing the antitryptic activity of the raw soybean meal. The experiment was therefore conducted to determine whether or not trypsin supplementation with navy bean diets would improve their nutritive value for rats.

Experimental

Preparation of diet and the details of rat feeding experiment were followed as described before. Trypsin (1:300) was supplemented at the expense of starch in the diets.

Results and Discussion

The results presented in Table 12 indicate that the trypsin supplementation (trypsin, 1:300) did not overcome the growth depression of rats fed raw navy beans. Moreover it was observed that trypsin supplementation was detrimental

TABLE 12.--Effect of supplementary trypsin on the growth of rats fed beans.

Exp. No.	Protein source	Average Change in wt.	Average Food Intake	Protein Efficiency Ratio (PER)
		g	g	
1	Raw beans	-12.8	88	100% mortality
2	Raw beans +1% trypsin	-11.4	80	100% mortality
3	Raw beans +2% trypsin	-11.0	82	100% mortality
4	Raw beans +3% trypsin	-10.8	76	100% mortality
5	Autoclaved beans	32.0	210	1.58
6	Autoclaved beans + 1% trypsin	25.0	190	1.31
7	Autoclaved beans + 2% trypsin	16.0	148	50% mortality
8	Autocalved beans + 3% trypsin	- 7.2	84	100% mortality

to rats receiving the autoclaved bean diet. These observations are consistent with those of Brambila et al. (50) who reported the detrimental effects of trypsin supplementation on the growth of chicks receiving either raw or heated soybean meal.

Part II

It has been reported (9, 51) that soaking prior to heat treatment is essential for normal growth of rats fed beans (Phaseolus vulgaris). The study was undertaken to examine the beneficial effect of soaking the beans on the growth of rats.

Experimental

The beans were soaked overnight in tap water, dried before a fan at room temperature and ground into flour. The details regarding the preparation of diet and rat feeding experiment were described in the first chapter.

Results and Discussion

The data in Table 13, however, showed that soaking the beans did not improve their growth promoting effects. This was true whether raw or autoclaved navy beans were used or when methionine (DL-methionine, 0.6%) was added to the ration. The importance of hemagglutinin as a causative factor for toxicity of the variety of beans belonging to P. vulgaris has been reviewed by Liener (29). It was stated

TABLE 13.--Effect of feeding soaked beans on the growth of rats.

Exp. No.	Protein Source	Average change in wt.	Average food intake	Protein Efficiency Ratio (PER)
1	Casein	82.0	283	2.90
2	Raw Beans	-14.0	92	100% mortality
3	Autoclaved beans	43.0	260	1.65
4	Soaked raw beans	-14.0	98	100% mortality
5	Soaked and autoclaved beans	46.0	272	1.69
6	Raw Beans Plus methionine	-11.2	94	50% mortality
7	Autoclaved beans plus methionine	84.0	288	2.95
8	Soaked beans plus methionine	-10.5	102	-1.03
9	Soaked and autoclaved beans plus methionine	89.0	291	3.06

that "if a hemagglutinin is responsible for the toxicity of raw kidney beans," as postulated by Honavar et al. (9) and Jaffe (51), "then it must follow that the hemagglutinin can only be destroyed in the raw meal by preliminary soaking followed by autoclaving." The fact that autoclaving the beans alone destroyed the hemagglutinating activity further supports that in navy beans there might be present a toxic factor (s) other than hemagglutinin.

Summary

The supplementation of trypsin on the growth of rats receiving raw bean diet was ineffective. A level of 2.0% and especially a level of 3.0% of a trypsin powder was "toxic" to the rats fed autoclaved bean diet. Preliminary soaking prior to autoclaving did not improve the growth promoting effect of navy beans. The low nutritive value of navy beans appears to be due to the presence of "toxic" factors(s) other than trypsin inhibitor and hemagglutinins.

CHAPTER VII

GROWTH INHIBITION OF RATS BY VARIOUS FRACTIONS OF THE BEANS

Recently Liener (29) reviewed the literature concerning the toxic factors present in edible legumes and indicated the importance of trypsin inhibitor and hemagglutinins as a cause of the low nutritive value of legume seeds. Bowman (4) has shown the presence of partially heat labile trypsin inhibitor in navy beans and suggested that its presence may account for the poor nutritive value of raw navy beans. However, no attempt has so far been made to isolate the navy bean trypsin inhibitor and study its effect on the growth of animals. Rigas and Osgood (5) purified the hemagglutinin from navy beans and reported that it is non-toxic to the animals. On the other hand, Honavar et al. (9) observed a definite growth inhibition of rats fed purified hemagglutinins from kidney beans and black beans. In the present investigation different fractions were obtained from navy beans and feeding experiments were conducted to determine whether a particular fraction having either trypsin inhibitor activity or hemagglutinating activity has any effect on the growth of rats.

Experimental

Fractions were isolated from raw navy beans by a technique outlined by Honavar *et al*. (9) as shown in Fig. 3. The isolation procedure was carried out in the cold at 4°C unless otherwise mentioned. Nitrogen content of each fraction was determined by micro-Kjeldahl method as described before.

Trypsin inhibitor activity was determined by the casein digestion method of Kunitz and hemagglutinating activity by the method of Liener as described before.

The method of Lowry *et al*. (52) was used for the protein determination. Reagents A, B, C, and E were prepared as follows:

Reagent A: 2% Na_2Co_3 in 0.1 N NaOH.

Reagent B: 0.5% $CuSO_4$. 5 H_2O in 1% Na Tartrate.

Reagent C: 50 ml. reagent A + 1 ml. reagent B.

Reagent E: Phenol reagent (commercial Fishers diluted with an equal volume of water).

The procedure consisted of adding 5 ml. of reagent C to 1 ml. of a suitable aliquot of the sample. The mixture was allowed to stand for ten minutes at room temperature and 0.5 ml. of reagent E. was then added. The mixture was further allowed to stand for 30 minutes at room temperature. The color thus developed was read in a spectrophotometer at 500 mμ.

The standard protein solution of egg albumin (0 to 125 mcg.) was run along the sample solution.

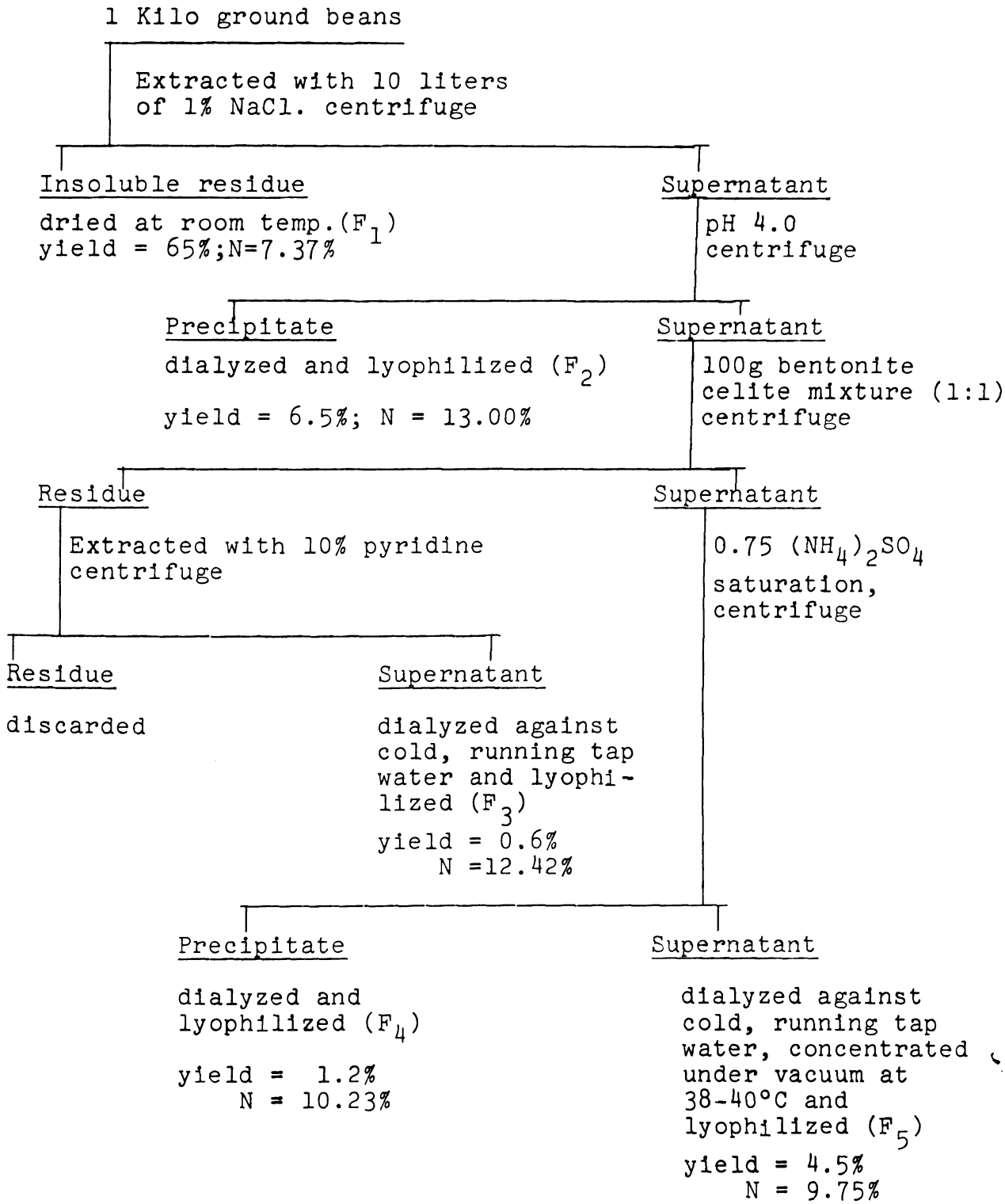

Fig. 3.--Preparation of Navy Bean Fractions

Preparation of diet and the details of rat feeding experiments were carried out as described previously. Raw or autoclaved navy bean flour (protein content 24%) was used as the source of protein in diets containing a level of 10% protein. The autoclaved beans were prepared by heating raw bean flour in the autoclave at 121° for 5 minutes. The isolated fractions were included in the autoclaved bean diet to determine their growth inhibitory effect. The same fractions were added to the raw bean flour separately and the mixture was then autoclaved to serve as an appropriate control.

Results and Discussion

Table 14 shows the hemagglutinating and trypsin inhibitor activities of various fractions isolated from raw navy beans. It can be seen from the table that the highest hemagglutinating activity lies in F_5 and F_4 while F_3 is devoid of hemagglutinating activity but contains the highest trypsin inhibitor activity. The low trypsin inhibitor activity in all other fractions and the low hemagglutinating activity in F_1 and F_2 may be due to incomplete separation during isolation procedure.

Results obtained by feeding the various navy bean fractions on the growth of rats are presented in Table 15. Rats fed the raw navy bean diet lost weight, consumed less

TABLE 14.--Hemagglutinating and trypsin inhibitor activities of various bean fractions.

	Hemagglutinating Activity HU/mg Protein	Trypsin Inhibitor Activity TIU x 10^{-3}/mg Protein
F_1	5.3	195
F_2	3.6	113
F_3	0.0	858
F_4	24.1	143
F_5	35.5	122

food and ultimately died within the experimental period of 28 days (Exp. 1A). On the other hand, rats fed the autoclaved bean diet gained weight and consumed a greater amount of food (Expt. 1B).

All fractions except F_5 when included in the autoclaved bean diet significantly inhibit the growth of rats as shown in Expt. 2A, 3A, 4A, and 5A (Table 15), the major growth inhibiting fraction being F_4. It is interesting to note that although F_5 contains the highest hemagglutingating activity and was included in the diet 2.5 times greater in quantity than that of F_4, it has no statistically significant effect on the growth of rats (Expt. 6A). It seems probable from the above experiment that hemagglutinating activity is not an essential factor for the growth depression in rats fed raw navy beans. It remains to be seen whether the hemagglutinating activity and the growth inhibiting activity of F_4 are due to one or more than one

TABLE 15. Effect of bean fractions on growth of rats.

Expt. No.	Protein Source (navy beans)	Average Change in Wt. g	Average Food Intake g	Protein Efficiency Ratio(per)
1A	Raw beans	-12.8	88	100% mortality
B	Autoclaved beans	33.0	220	1.50 ± 0.10
2A	Autoclaved beans + 30% F_1	15.7	125	1.27[a] ± 0.26
B	(Raw beans + 30% F_1) Autoclaved	51.0	258	2.37 ± 0.07
3A	Autoclaved beans + 5% F_2	35.0	240	1.44[a] ± 0.07
B	(Raw beans + 5% F_2) Autoclaved	56.3	266	2.10 ± 0.19
4A	Autoclaved beans + 1% F_3	13.0	184	0.70[a] ± 0.08
B	(Raw beans + 1% F_3) Autoclaved	43.0	247	1.69 ± 0.12
5A	Autoclaved beans + 1% F_4	- 4.0	94	-0.45[a] ± 0.16
B	(Raw beans + 1% F_4) Autoclaved	41.3	271	1.52 ± 0.04
6A	Autoclaved beans + 2.5% F_5	17.6	179	0.96[b] ± 0.14
B	(Raw beans + 2.5% F_5) Autoclaved	30.6	196	1.53 ± 0.21

[a]Highly significant $P < 0.01$, compared to appropriate treatment.

[b]Not significant $P > 0.05$, compared to appropriate treatment.

factors. The work of Jaffe (53) and that of Honavar et al. (9) indicate that growth inhibition of rats fed black beans or kidney beans is due to their hemagglutinin content. However, it appears that the nutritional significance of hemagglutinins should be established carefully especially in the light of very recent work of Funatsu (54) who separated the toxic activity and hemagglutinating activity of ricin.

It may be that there are two types of hemagglutinins--one is toxic and the other one non-toxic. It is likely that the F_5 fraction may be identical to the non-toxic hemagglutinin isolated by Rigas and Osgood (5).

Experiment 4A (Table 15) shows that navy bean trypsin inhibitor has a deleterious effect on the growth of rats. Growth depression observed by the inclusion of F_1 (Expt. 3A) in the diet is very difficult to rationalize. It is possible that the residual trypsin inhibitor activity present in them may account for the observed growth inhibition of rats. Recent studies of Saxena et al. (55) on raw soybean meal indicated that the major growth inhibiting factor for chicks resides in the water insoluble residue devoid of trypsin inhibitor activity. It may be that the growth inhibiting factor present in F_1 (insoluble fraction) is similar to that of soybean meal. The results of Rackis et al. (56) also indicated that there is no direct correlationship between trypsin inhibitor activity of different soybean fractions and their influence on growth inhibition or pancreatic hypertrophy of rats.

In the light of the above discussion the results presented in Table 15 could be explained in a different way. It can be suggested that neither the hemagglutinin nor the trypsin inhibitor are toxic but there is a toxic material that was scattered throughout all of the navy bean fractions in differing amounts. Fraction F_4 appears to contain the highest and Fraction F_5 the least concentration of that toxic material.

Summary

Five different fractions were isolated from raw navy beans and their effect on the growth of rats was studied. All these fractions except one which had the highest hemagglutinating activity significantly inhibited the growth of rats. F_4 was shown to be the major growth inhibiting and the next highest hemagglutinating activity content fraction. Growth inhibitory effect of F_3 and possibly of F_1 and F_2 on rats was attributed to the trypsin inhibitor activity. The possibility of the presence of a toxic factor other than hemagglutinin and/or trypsin inhibitor in navy beans is discussed.

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APPENDIX

The vitamin diet fortification mixture used in the preparation of diet supplies the following vitamins in mg/100 g of diet: vit. A concentrate (200,000 units per gram), 9.0; vit. D concentrate (400,000 units per gram), 5.0 alpha tocopherol, 10.0; ascorbic acid, 90.0; inositol, 10.0, choline chloride; 150.0; menadione, 4.5; p-aminobenzoic acid; 10.0 niacin, 9.0; riboflavin, 2.0; Ca pantothenate, 6.0; biotin, .04; folic acid 0.18; vit. B_{12}, .0027.

The standard error, s, is the square root of the variance, S^2 and it was calculated as follows:

$$S = S^2$$

$$S^2 = \frac{\varepsilon x^2 - \left(\frac{\varepsilon x^2}{n}\right)}{n - 1}$$

where x = observed measurement

n = numbers of observations in samples.

The significance test was carried out by "student's" t- distribution test described as follows:

$$t = \frac{(\bar{X}_1 - \bar{X}_2)}{S_{x1} - S_{x2}} = \frac{(\bar{X}_1 - \bar{X}_2)}{\sqrt{\frac{S_x^2}{n_1} + \frac{S_x^2}{n_2}}} = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{S_x^1 \left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$

where $\bar{X}$ = mean of a sample of measurements x.

n_1 = size of the sample from which $\bar{X}_1$ is computed.

n_2 = size of the sample from which $\bar{X}_2$ is computed.

S_x^2 was caluclated by the formula:

$$S_x^2 = \frac{x_1^2 - \frac{(x_1)^2}{n_1} + x_2^2 - \frac{(x_2)^2}{n_1}}{n_1 + n_2 - 2}$$

Where the symbols have usual meanings as described before.