EFFECTS OF FEEDING COTTONSEED PROTEIN ON THE IN VITRO AMINO ACID INCORPORATION OF RAT LIVER POLYRIBOSOMES

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CHAPTER I

INTRODUCTION

As a protein source for human consumption, cottonseed flour may contribute toward alleviation of protein malnutrition found around the world. The realization that cottonseed flour could provide a supplementary protein supply and the omnipresent interest in improving nutritional quality has stimulated interest in availability of the cottonseed flour through new methods of growing and processing the cottonseed (54, 34). The development of a glandless cottonseed is one result of this continuing effort (34). The quality of the protein contained in cottonseed flour has been reported to be of higher biological value than several other popular protein sources (40).

Rationale

The quality of protein is determined by the essential amino acid content and the proportion of these constituents. Although protein is synthesized in other body organs, the liver is the major organ of protein synthesis. A factor that may influence the quantity of protein synthesis by the liver is the quantity and/or quality of the protein consumed in the diet (26). Consequently, a reasonable hypothesis would predict that the relative supply of amino acids will affect the mechanisms of protein biosynthesis.

Methodology is currently available to assess the effect of protein intake on protein synthesis, thus enabling an examination of the effect(s) of amino acid supply on the subcellular components engaged in this synthesis. Potentially, therefore, the detection of subtle effects due to an imbalance in amino acids should be possible.

Polyribosomes or polysomes which are the site of polypeptide chain formation are composed of ribosomes attached to messenger RNA (mRNA). Amino acid availability alters the rate of protein synthesis and thereby the rate of association and disassociation of the ribosomes with the mRNA (88). Consequently, the lack of one amino acid required in the peptide sequence could alter polysome stability (88) as well as activity. In cottonseed protein, methionine and lysine are limiting amino acids (15, 23); therefore, a diet in which cottonseed flour is the only source of nitrogen could have an effect on protein synthesis due to this imbalance.

Objective

The overall objective of this research project was to study the effect of feeding cottonseed protein as the sole source of nitrogen on protein synthesis at the subcellular level, and compare the effect(s) with diets lacking in methionine or lysine. Specifically, this study was conducted to:

1. determine effect(s) of these diets on *in vitro* amino acid incorporation by rat liver polyribosomes as measured by

incorporation of ^{14}C-labelled amino acids; and

2. investigate any alterations in the relative quantity of protein or RNA found in the polyribosomes.

CHAPTER II

REVIEW OF LITERATURE

Amino Acid Imbalance

The importance of the relationship between dietary amino acids and the amino acid requirements of a particular organism has been recognized since 1914 (10, 43). Ordinarily, dietary amino acid proportions, especially in cereal products, deviate from those actually required by the organism (26); therefore, nutritional quality of a protein is a direct function of the amount of the growth-limiting amino acid available to the organism and is independent of amino acids which may be in excess.

The term 'amino acid imbalance' is defined as a disproportion of amino acids great enough to cause an adverse effect. This negative effect must be preventable by a supplement of the limiting amino acid, and no amino acid may be present in an amount sufficient to be considered toxic by itself (26). The conditions under which adverse effects are observed are for the most part quite uncommon and are usually experimentally contrived to create an imbalance greater than might be encountered naturally. These adverse effects, as stated by Harper et al. (26), may occur in human subjects whose susceptibility to an amino acid load is increased because of liver damage, protein malnutrition, or a genetic defect in amino acid metabolism. This suggests that the ingestion of disproportionate amounts of amino acids may result in adverse

effects to an organism when homeostatic mechanisms for the regulation of blood and body fluid amino acid concentrations are deficient, defective or artificially overloaded (26).

The basic negative effects of an amino acid imbalance depression of growth and food intake (6, 25, 55, 66), fatty infiltration of the liver (4, 5, 29), alteration in body composition (e.g. body fat and water content) (24), and lowered efficiency of amino acid utilization (24, 41). The severity of the effects varies with the nature and degree of the amino acid disproportion, the nutritional adequacy of the diet, i.e. vitamin and mineral content, and the age and physiological state of the organism (26).

In general, the use of the term imbalance has been restricted to disproportions involving only essential amino acids. However, there have been reports of growth depression caused by surpluses of non-essential amino acids (6, 21, 67, 77); usually much larger amounts are needed than with essential amino acids. It is not known if adverse effects due to imbalances caused by mixtures of essential amino acids have the same basis as those due to additions of non-essential amino acids. There is a need for further research in this area as cited by Harper et al. (26) in their review of this subject.

Nutritional Studies of Diets Lacking Amino Acids

Studies have been conducted on the morphological and biochemical changes in rats fed diets lacking a single essential amino acid (69, 70,

71). To compare the effect of feeding a diet devoid of an amino acid to an amino acid imbalanced diet, the control group should be fed a diet containing the same quantity of the limiting amino acid as the experimental diet under conditions where other amino acids are not in excess. Therefore, a protein free diet, rather than an adequate diet, should be used as one of the control groups to provide additional, appropriate comparison. In two such studies, by Sidransky and Rechcigl (71) and Ousterhout (56), the protein-free group of animals did as well as, or better than, the group fed a diet lacking a particular amino acid. Comparisons between animals fed an amino acid-devoid diet and those fed a nutritionally complete diet reflect the effects of an amino acid deficiency rather than an imbalance. The studies of Harper et al. (26) in which the differences are due to variations in the content of the limiting amino acid resemble comparisons between animals fed an imbalanced diet and those fed a complete diet.

Both a dietary amino acid imbalance and a diet devoid of one essential amino acid, when fed to rats, are reported to create a depressed food intake (66, 71). It is postulated that the low food intake of rats eating a diet severely deficient in an amino acid is a response to a homeostatic mechanism. Normally the homeostatic mechanism curtails dietary intake, thus controlling overeating which would result in an accumulation of body fluids (1). These fluids contain excessive amounts of amino acids which cannot be used for protein synthesis. This accumu-

lation can, in turn, lead to adverse effects, i.e. alteration in plasma amino acid patterns and fluid retention, as observed in animals force-fed a diet lacking in one amino acid (25). The similarity of the food intake responses and the plasma amino acid patterns of rats fed amino acid imbalanced diets suggests that the signal is the same for both conditions and is in some way related more directly to the abnormal plasma amino acid pattern (47).

Amino Acid Incorporation by Polyribosomes

Dietary amino acid content can also manifest an effect at the subcellular level. One means for evaluating the effects at this level would be to examine the *in vitro* protein synthesis by the polyribosomes.

Polyribosomes are aggregates of ribosomes held together by strands of messenger RNA (mRNA) which code for the amino acid sequences of proteins (88). Polyribosomes are considered to be the functional units of protein biosynthesis (85). Wettstein *et al.* (85) indicated that these aggregates are the working particles and that the aggregate structure is necessary for protein synthesis *in vivo* and for amino acid incorporation *in vitro*. Munro and co-workers (42) were able to show that a polysome structure was not essential for amino acid incorporation into protein and that single ribosomes, suitably attached to mRNA, could be active. Even though Munro's work has been verified, there still appears to be preferential in larger polysomes (up to 8 ribosomes/polysome).

Several reports indicated that in starved animals the ingestion of either a protein or a complete amino acid mixture results in a rapid

increase in the degree of hepatic polyribosomal aggregation and rate of protein biosynthesis (6, 22, 30, 71). However, omission of one essential amino acid (tryptophan) from the diet was able to inhibit the change in the polyribosomal pattern and the stimulation of amino acid incorporation (88). In livers of rats fed a tryptophan-free diet, a larger percentage of the cytoplasmic RNA was associated with the oligosomes, free ribosomes and ribosomal sub-units, as compared with controls (30). These data led to the conclusion that a deficiency in amino acid supply results in a breakdown of the polyribosomes eventually causing a loss of cytoplasmic RNA which can be correlated with a decreased rate of protein biosynthesis (22). Wannemacher et al. (84) concluded from their work using an amino acid-deficient (6 percent casein) diet that, in the liver, dietary amino acids regulate protein synthesis by influencing both the ability of a unit of polyribosomes to incorporate amino acids into protein and the number of ribosomes per cell. The former process responds rapidly to the particular supply of amino acids and is independent of RNA synthesis; whereas, the latter proceeds at a slower rate and depends on the synthesis of new RNA (84).

Henshaw and colleagues (30) quantified the factors which control protein synthesis in normal and starved rats. Through their study, they aided in the identification of the primary mechanisms which modulate the synthesis rate per unit of total ribosomes for the liver. Both the fraction of ribosomes in polyribosomes and the synthesizing activity of these ribosomes can be measured reliably for liver tissue. These

investigators assessed the extent to which alterations in these two factors account for the decreased rate of protein synthesis per ribosome during fasting. They also looked for rate differences in rates among non-fasted animals. In non-fasted animals, the proportion of polyribosomal ribosomes was always 90-95 percent of the total ribosomes. During fasting the proportion of monomeric (single) ribosomes increased, and a large increase in dimers (aggregates containing two ribosomes) was also found (19). The proportion of polyribosomes varied considerably in rats fasted for 3½ days, ranging from 32-84 percent as opposed to the normal values of 90-95 percent. Henshaw et al. (30) reported that results between polysomes from non-fasted animals (tested in vitro) suggest that the large differences noted in vivo are due to supernatant factors rather than to alterations in the polyribosomes themselves. With fasted animals, the decreased polyribosomal activity as measured in vivo was also reflected in in vitro measurements. The decrease in protein synthesis in the liver of fasted animals was found to be due to approximately equal decreases in three things: (a) ribosome content, (b) polyribosome activity, and (c) the proportion of ribosomes in polyribosomes (14).

The ability of ribosomes to incorporate amino acids into protein in vitro has been correlated with the quality of the protein source. These observations have been made not only in liver ribosomes, but also in the ribosomes prepared from skeletal muscle. In one such study, Omstedt and von der Decken (51), ribosomes were obtained and compared

from groups of rats receiving each of the following treatments: (a) a protein-free diet for 5 days followed by a high-protein diet for 16.5 hours containing, as the protein source, either casein, gelatin or wheat gluten, (b) a high-protein diet containing either casein, gelatin, wheat gluten and, (c) one containing protein as polished rice for 6 days. The level of ^{14}C-amino acid incorporation relative to RNA was somewhat higher when the protein source given for 16.5 hours was a good quality protein such as casein. It was lower with gelatin or wheat gluten, but fine discrimination between proteins was not considered to be feasible with this system. In the rats given the protein-containing diets for 6 days, the differences were more pronounced and the amino-acid incorporating activity was correlated with the biological value of the protein (51). von der Decken and Omstedt (82) also measured the amino acid incorporating activity of the skeletal muscle ribosomes in rats which were fed a diet containing either 10-percent or 20-percent protein of varying biological values for 6 consecutive days. In rats fed the 10-percent protein diets there was no difference in the amino acid incorporating activity of the ribosomes/mgRNA between the four dietary proteins studied. However, when the radioactivity data were calculated per milligram protein, and thus per unit wet-weight of skeletal muscle, a correlation was found between the amino acid incorporating activity and the biological value of the proteins. A more pronounced correlation was found when the rats were fed the 20-percent protein diet (82).

In a more recent study (52) some aspects of the effects of various dietary proteins and amino acid mixtures on the protein-synthetic ability of ribosomes from skeletal muscle and liver of rats was investigated. The metabolic utilization of a high-quality protein was compared with that of an amino acid mixture which had a composition that gave optimal utilization. A low-quality protein and a mixture deficient in a single amino acid were also compared. The level of ^{14}C-amino acid incorporation relative to ribosomal RNA was similar for casein supplemented with methionine and for a complete amino acid mixture with the composition of whole-egg protein. In skeletal muscle, but not in liver, the ribosomal activity was less than that obtained with wheat gluten. Conversely, there was (per wet-weight of tissue) a significant increase after feeding with the complete amino acid mixture. There was a significant decrease in activity after feeding with amino acid mixtures deficient in lysine, methionine, or trytophan. Activity per wet-weight of both tissues was less than that obtained with wheat gluten.

Distribution of Free and Membrane-Bound Ribosomes in Rat Livers

As opposed to previously discussed studies involving total ribosomal preparations, studies of separated free and membrane-bound ribosomes have been conducted in order to elucidate additional specific aspects of dietary effects.

Ribosomes account for about 80-90 percent of the total RNA in rat liver (7, 8, 32). In all mammalian cells, cytoplasmic ribosomes are present in at least two forms, either attached to membranous structures or free in the cytoplasmic matrix (57). Membrane-bound and free ribosomes provide about 32 and 68 percent, respectively, of the total liver content of ribosomal RNA (rRNA) in normal adult rats (60). Numerous studies have focused on the mode of attachment of liver ribosomes to membranes (9, 59, 65), possible intrinsic structural differences between the two groups of ribosomes (36), metabolic differences (9, 37, 49), and functional significance of their partition between membranes and the cytoplasmic matrix (2, 31). Alterations in the relative proportions of membrane-bound and -free ribosomes of rat liver have been associated with the age of the animal (12, 50), regeneration following partial hepatectomy (11), and treatment with various chemical agents (61, 76). Reports on the effects of the nutritional state of the animal on the partition of liver ribosomes between membrane-attached and cytoplasmic-free pools are conflicting and not adequately defined. When a rat is starved (16, 19, 78) or fed a protein-free diet (7, 46), the liver loses RNA. Similar observations have been noted in rats fed a low protein diet (18, 52, 84) or a diet deficient in one or more essential amino acids (42, 48). Munro (47) believes that this loss in liver RNA is due to breakdown of the rough-endoplasmic reticulum and intact ribosomes with elevation in the ratio of free versus bound ribosomes (26, 84) as well as a significant reduction in the proportion of total liver RNA derived

from the ribosomes (84). In contrast, prolonged starvation of rats to reduce liver RNA content by about 50 percent was found to produce no alterations in the relative distribution of the cytoplasmic ribosomes (8, 29, 32). Enwonwu (16) studied the effect of prolonged feeding of inadequate protein diets of growing rats at various stages of development. He found extensive loss of liver RNA with hardly any alteration in the normal proportion of liver RNA derived from the ribosomes. This finding is in conflict with the observations of Wannemacher, Cooper and Yatvin (84) who reported yields of approximately 41 and 24 percent of the liver RNA from total cytoplasmic ribosomes of control and protein-deficient rats, respectively. Discrepancy between the two studies, findings, sets of data may be attributed to differences in techniques used (16).

With the advent of new biochemical techniques, the evaluation of nutritional problems can be examined with increasing depth in order to provide not only an understanding of the gross affects of dietary deficiencies but the actual mechanisms by which these manifestations occur. In this study, the effect of specific deficient diets on in vitro amino acid incorporation by rat liver polysomes were investigated. The selection of the animal model and some procedures and protocols were taken with the bulk of the literature in mind so that results obtained could be more directly compared and discussed in terms of the existing literature; however, specific techniques were chosen to provide sufficient depth to the investigation so as to enable a structure-activity correlation should the results permit.

CHAPTER III

METHODS

Animals

Rats (=157g) were supplied by ARF Sprague Dawley Company, Madison, Wisconsin. Specific pathogen free (SPF) male albino rats were quarantined until microbiological surveillence and observation substantiated a healthy condition. All 30 animals were housed individually in a room with controlled temperature (25 ± 1 ^{o}C) and humidity and a 12-hour light/dark cycle. Water and food, whether standard lab chow (during quarantine) or special diets, were supplied ad libitum. Additional rats (approximately 85) were used in the preparation of cell sap and preliminary experiments.

Diets and Feeding Procedure

Following quarantine, the animals were placed on an equilibration diet containing 10 percent protein for a 3-day period prior to being given the experimental diet. The purpose of the equilibration diet (72) (see Table 1 for specific composition) was to deplete the body stores of protein so that any effect of the experimental diets would be evident following a relatively short feeding protocol. The 10-percent protein diet for 3 days is able to create a standard depletion without noticeable body weight loss (72).

TABLE 1

COMPOSITION OF THE EQUILIBRATION DIET

Ingredient	g/100 g of diet
Vitamin free casein	10.9
Corn starch	48.9
Sucrose	24.5
Salt Mixture R. H.*	5.0
Vitamin diet fortification mixture *	0.5
Choline chloride	0.2
Corn oil	10.0

*For further details see Appendices B and C.

Following the equilibration period, the animals were randomly divided into five groups. Consideration was given to grouping so as to have average group weights which were similar. Each experimental group contained five animals, and the control group contained ten animals. The source of protein for each of the groups was either casein (control group), methionine-free amino acid mixture, lysine-free amino acid mixture, or glandless cottonseed flour, while one group consumed a diet devoid of protein. All groups, except the protein free group, received diets containing 14.9-percent protein by weight. Food intake and weight gain were recorded. The composition of the five diets is shown in Table 2. A comparison of amino acid content of casein, cottonseed, and ovalbumin is given in Appendix A.

Sacrifice Procedure

At the end of the 6-day experimental feeding period, one rat from each experimental group and two rats from the control group were sacrificed on the next 5 consecutive days. Experimental diets and water were continued *ad libitum* until sacrifices were complete. Each animal was lightly anesthetized and sacrificed by cervical dislocation and immediately prepared for the liver perfusion as described below.

Preparation of Liver Tissue

Preparation of polysomes from liver were carried out essentially as described by Ramsey and Steele (60). All materials used in the pre-

TABLE 2

COMPOSITION OF THE CONTROL AND EXPERIMENTAL DIETS

Ingredient	g/100 g of diet				
	Group 1 Control (Casein)	Group 2 Methionine-free (Met$^-$)	Group 3 Lysine-free (Lys$^-$)	Group 4 Cottonseed flour* (CS)	Group 5 Protein-free (Pro$^-$)
Protein source	18.2	14.9	14.9	25.4	-
Corn starch	44.1	45.8	45.8	40.5	53.2
Sucrose	22.0	23.6	23.6	19.4	31.1
Salt mix**	5.0	5.0	5.0	5.0	5.0
Vitamin mix***	0.5	0.5	0.5	0.5	0.5
Choline chloride	0.2	0.2	0.2	0.2	0.2
Corn oil	10.0	10.0	10.0	10.0	10.0

*Glandless cottonseed flour.
**See Appendix B.
***See Appendix C.

parations of liver tissue and subsequent experimental procedures are described in Appendix D. The livers were perfused *in situ* via the portal vein with ice-cold 250 mM sucrose-1-mM $MgCl_2$ (40-50 ml). The livers were then quickly removed and placed in ice-cold perfusion medium and taken into the cold room. All subsequent operations were performed at 0-to4 ^{o}C. Livers were trimmed, blotted, weighed, and finely minced with sharp scissors in order to cut up the fibrous tissue which can decrease the effectiveness of homogenization.

Homogenization

The minced tissue was homogenized with three volumes (w/v) of polysome buffer (50 mM Hepes, pH 7.4 at 4 ^{o}C, 250 mM KCl, 5 mM $MgCl_2$, and 3 mM glutathione) containing 250 mM sucrose in a glass homogenizer with 10 strokes of a motor-driven Teflon pestle (0.006 - 0.009 inch clearance, size C, A. H. Thomas Co.) rotating at 1750 rpm. A minimum of 95-percent cell breakage is obtained by use of this procedure according to Ramsey *et al.* (60).

Separation of Free and Bound Polysomes

Aliquots of the homogenate (20 ml) were placed into 25-ml flasks to which 50 units of alpha amylase per ml of homogenate was added. This was incubated on ice for 15 min to digest the glycogen present in the livers of non-fasted animals. Samples containing 17.5 ml were centrifuged at 740 X g for 2 min, and then the centrifugal force was increased

to 131,000 X g and maintained for 12 min in a Beckman SW 27.1 rotor. The supernatant was decanted and saved for purification of free polysomes. The pellet (particulate fraction) was suspended with 4 strokes of a glass-Teflon homogenizer using a size B pestle in 50-percent cell sap (S_3) modified to contain a final concentration of 250 mM KCl. One-ninth volume of 10-percent (w/w) Triton X-100 was added, then the mixture was homogenized with 3 strokes at 1750 rpm and centrifuged at 1470 X g for 5 min to sediment nuclei. The resulting supernatant was decanted and mixed with one-ninth volume of 13-percent (w/w) sodium deoxycholate to release bound polysomes from membranous material.

Purification of Polysomes

Aliquots of 3 ml from the supernatants containing either free polysomes or solubilized bound polysomes were layered over discontinuous gradients composed of 3 ml each of 1.38 and 2.0 M sucrose, plus cell sap. These solutions were prepared by the addition of appropriate amounts of cell sap to a 2.3 M stock solution of sucrose made up in polysome buffer. They were then centrifuged at 174,000 X g for 20 hr (50 Ti rotor, Beckman) to pellet polysomes. After centrifugation, the supernatant layers were decanted and discarded. The tube walls were washed with cotton swabs dampened with polysome buffer and then dried. The polysomal pellets were suspended in polysome buffer and gently homogenized by hand with a Teflon-glass homogenizer. Aliquots of the suspension was taken for chemical analysis. Protein was determined by the method of

Lowry et al. (38), and RNA was determined from the optical density at 260 nm; one optical density unit at this wave length was taken to correspond to 50 ug of RNA (equivalent to an $E^{1\%}_{1cm}$ at 260 nm of 200) (37).

Preparation of Cell Sap (S_3)

Cell sap for use as a source of natural RNase inhibitor (7) was prepared from the liver of fasted rats. A 50-percent (w/v) homogenate was prepared using the polysome buffer, and centrifuged at 368,000 X g for 95 min or its g-min equivalent. The lipid portion was suctioned off, and the upper three-quarters of the supernatant was used. This portion retains full RNase-inhibitor activity for at least 2 months at -20 ^{o}C and was shown to be ribosome-free when analyzed by the polysome purification procedure (60).

Amino Acid Incorporating System In Vitro

The assay medium (1.0 ml/assay tube) for amino acid incorporation in vitro contained the following in u moles (additional specifics are stated in Appendix D): sucrose, 175; Hepes buffer (pH 7.6 at 37 ^{o}C), 50; KCl, 35; $MgCl_2$, 2.75; ATP (magnesium salt), 1; GTP (sodium salt), 0.1; creatine phosphate (disodium salt), 20; and a mixture containing 0.1 u moles of each of the following L-amino acids: alanine, aspartic acid, arginine, cystine, glycine, glutamic acid, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalaxine, proline, serine, tryptophan, tyrosine, and valine. Each assay tube contained

3.5 ug creatine phosphokinase (163 units/mg of protein with creatine as substrate), 1.0 uCi of ^{14}C leucine (50 uCi/ml; 320 mCi/m mole), and 0.4 ml (12 mg of protein), cell sap (about a 50-fold excess over rRNA). The medium was preincubated for 2 min at 30 ^{o}C, at the end of which, polysomal suspension (0.24 mg of rRNA, assuming an $E^{1\%}_{1cm}$ at 260 nm of 200) was added. For cell sap blanks, polysome suspension buffer (pH 7.3 at 30 ^{o}C) was added instead of polysomes. Cell sap was prepared from non-fasted rats and was chromatographically treated (Sephadex G-10) to remove endogenous amino acids (33). Incorporation of radioactive amino acids into radioactively tagged trichloroacetic acid insoluble products was determined after incubation for 10 min at 30 ^{o}C. Aliquots of the incubation mixture (100 ul) were transferred to filter paper disks and processed according to Mans and Novelli (39). The processed disks were placed in 20 ml Scintiverse and counted for radioactivity in a Packard Tri-Carb Scintillation Spectrometer. The radioactivity is expressed as cpm/mg rRNA.

Measurement of Polysome Size Distribution

Aliquots of polysomal suspensions (7.5 A_{260} units) were layered over 15-27.8 percent (w/w) isokinetic sucrose gradients containing 10 mM Hepes (pH 7.4) at 4 ^{o}C, 250 mM KCl, 5mM $MgCl_2$, and 0.5 mM EDTA and centrifuged at 131,000 X g for 105 min at 2 ^{o}C (60). Sucrose solutions were prepared from commercial sucrose that had been treated with acid-washed charcoal to remove RNase and substances absorbing at 254 nm (79).

After centrifugation, the gradients were monitored continuously at 254 nm with an automatic analyzer and recorder (ISCO, Lincoln, Nebraska).

Sacrifice Schedule

Animals were sacrificed over a 5-day period following the initial 6-day experimental feeding period. This schedule enabled the samples from sacrificed animals to be processed immediately which was essential for the isolation of undegraded polysomes (33). At the same time, the schedule was selected to equalize, although not eliminate, the day-to-day variances that would have arisen from sacrificing a group on a given day rather than one animal from each group on a given day. Although it is recognized that elimination of the day-to-day variable could have only resulted from a total sacrifice and processing on a single day, appropriate statistical analysis design can correct for this day-variable by analyzing the day-variable component and mathematically adjusting the total effect accordingly so as to focus on the treatment effect.

Statistical Analysis

An analysis of variance was performed on each of the parameters studied i.e. ^{14}C amino acid incorporation, RNA, protein and RNA/protein ratio for both the free- and the bound-polyribosomal fractions. When an effect was observed, the test of least significant difference was applied to determine the degree of variability.

A paired t-test was used to compare the differences between the mean ^{14}C activity of the bound- and free-polyribosomal preparations. These were considered to be statistically significant from zero at =0.05. These statistical procedures were taken from Statistical Principles in Experimental Design (86, 87).

CHAPTER IV

PRESENTATION AND DISCUSSION OF DATA

As stated in Chapter I, the purpose of this study was to determine the effect of feeding cottonseed protein as the sole source of nitrogen on the *in vitro* amino acid incorporation of rat liver polyribosomes. These results were compared with results obtained from animals which were maintained on a control diet in which the sole source of dietary nitrogen was supplied from casein, as well as groups of animals fed a lysine-free, methionine-free or a protein-free diet.

Comparison of Food Intake and Weight Gain

The animal selected for this study was male Sprague-Dawley rats of approximately 6 weeks of age. Prior to the start of the experiment, 30 animals were placed on an equilibration diet for a 3-day period. The equilibration diet was balanced in all necessary nutrients and contained 10-percent protein by weight; the composition is given in Table 1. Weight gain for all animals was similar during this period with a mean daily weight gain of 6 g per animal.

At the end of the equilibration period, the animals were randomly assigned to groups and caged individually. Each animal was given the experimental diet and water *ad libitum* throughout the initial 6-day feeding period. A comparison of the mean body weight by group as of the

start of the experimental feeding period, and changes in the body weights during this feeding period are presented in Table 3.

At the end of the 6-day feeding period of experimental diets, the methionine-free, lysine-free and protein-free groups had all exhibited a mean daily weight loss. The final weights of the animals in the control group and the cottonseed-flour groups showed an increase in body weight following the feeding period.

For the control and cottonseed-flour groups, the mean daily food intake was similar. The other three groups had depressed food intakes and a resulting loss of weight (Table 3). A comparison of food intake and weight gain for the casein and cottonseed groups, showed that although the cottonseed group ate more they did not grow as much as the casein group. Retarded growth, as well as depression in food intake are both found routinely in animals maintained on diets which contain an amino acid imbalance (24, 58, 64). A more detailed discussion of this topic is included in the Review of Literature (Chapter III).

In Vitro Amino Acid Incorporation

Following sacrifice, samples for *in vitro* amino acid incorporation were prepared as outlined in the Methods (Chapter III). This cell-free system was designed so that any variation in the incorporating ability of the system was due only to polysomes. All other components were constant between samples.

TABLE 3

COMPARISON OF FOOD INTAKE AND WEIGHT GAIN AMONG RATS IN THE CONTROL AND EXPERIMENTAL GROUPS

Diet	Mean Starting Weight (g)	Mean Daily Weight Gain (g)	Mean Daily Intake (g)	Ratio of Average Daily Weight Gain to Daily Intake
Control	160	5.6	15.0	0.37
Met-	157	-1.5	7.8	-
Lys-	158	-0.5	12.6	-
CS	157	4.9	15.9	0.31
Pro-	155	-2.5	5.8	-

The ^{14}C amino acid incorporation of the rat liver polysomes obtained from rats maintained on the different protein sources is presented in Appendix E and F. This synthesizing activity is expressed per mg of ribosomal RNA (rRNA) and is based on an $E^{1\%}_{1cm}$ at 260 nm of 200. The mean counts per minute (cpm) and the standard deviation for each group is included in Table 4. There was a linear relationship between the 5- and 10-min incubation times for all groups (Fig. 1). This was true for both the free and bound polyribosomes, which indicates that the system was functioning properly, and is in agreement with the work of other investigators (60).

Analysis of variance among groups showed a treatment effect (Appendix G-J). Further analysis showed a statistically significant difference in the amino acid incorporating ability among all groups (Table 5). This was determined by the use of the test of least significant difference as described in Chapter II. The casein group, which served as the control, consistently had the highest rate of incorporation for both the free and the bound polysomes. Although the bound polyribosomes had a slightly higher rate of incorporation as compared to the free polyribosomes for all groups the differences were not statistically significant at $p>0.05$ with the exception of the control group. Even though the among group differences were statistically significant, the differences between groups varied. The cottonseed group had the second highest rate of incorporation with less difference between the cottonseed group and the casein (control) group than between the control and any

TABLE 4

AMONG GROUP COMPARISON FOR ^{14}C LEUCINE INCORPORATION OF RAT LIVER POLYRIBOSOMES

Group	Incubation Time	No. Animals/ group	Mean * (cts/min/ mgRNA)	Standard Deviation
Casein		10		
Bound	5 Min*		26612.	3033
	10 Min		52258.	3721
Free	5 Min		22165.	3031
	10 Min		47826.	6727
Cottonseed		5		
Bound	5 Min		23681.	1604
	10 Min		47826.	3629
Free	5 Min		21258.	2713
	10 Min		42750.	7676
Lys-		5		
Bound	5 Min		14904.	3866
	10 Min		29403.	4751
Free	5 Min		13667.	2955
	10 Min		25428.	4183
Met-		5		
Bound	5 Min		7936.	562
	10 Min		18197.	1807
Free	5 Min		6989.	1167
	10 Min		16252.	1673
Pro-		5		
Bound	5 Min		4256.	1373
	10 Min		8714.	2884
Free	5 Min		4070.	1370
	10 Min		8434.	2940

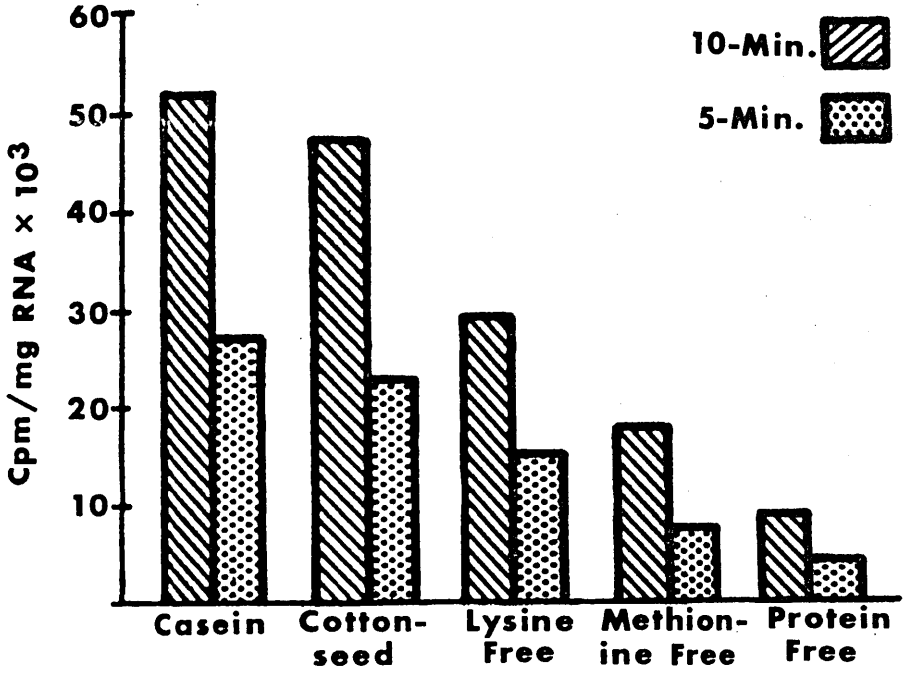
*All group means were found to be statistically significant at $p<0.05$.

TABLE 5

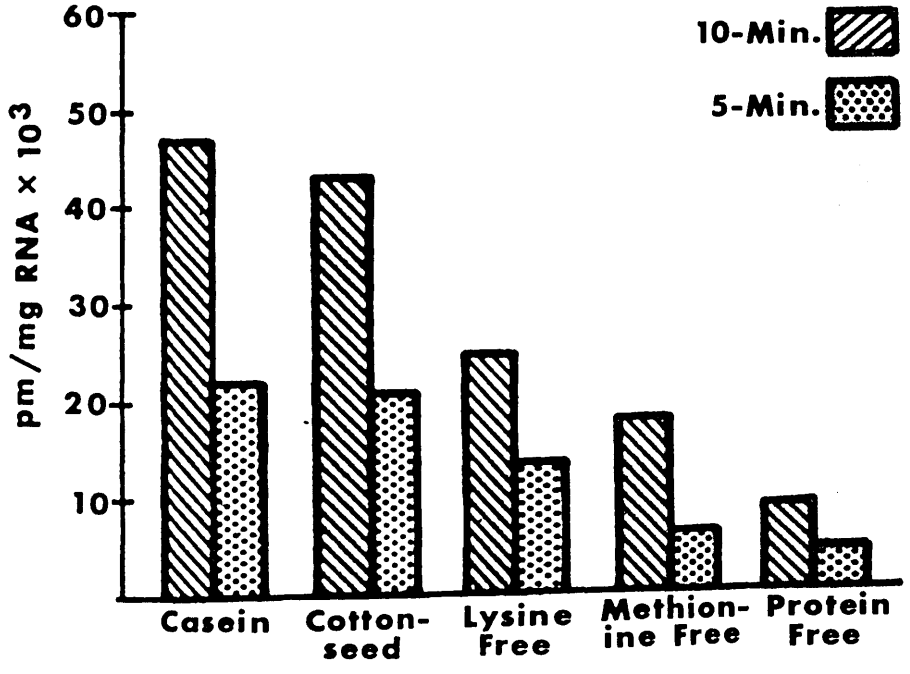
INFLUENCE OF DIETARY AMINO ACID COMPOSITION ON IN VITRO INCORPORATION OF ^{14}C LEUCINE INTO RIBOSOMAL PROTEIN

Group	5 Min. Incubation		10 Min. Incubation	
	Bound (Mean ± S.D.)	Free (Mean ± S.D.)	Bound (Mean ± S.D.)	Free (Mean ± S.D.)
Casein (n=10)	26.6 ± 3.0	22.2 ± 3.0	5.23 ± 3.7	47.8 ± 6.7
Cottonseed (n=5)	23.7 ± 1.6	21.3 ± 2.7	47.8 ± 3.6	42.8 ± 7.7
Lys^- (n=5)	14.9 ± 3.9	13.7 ± 3.0	29.4 ± 4.8	25.4 ± 4.2
$Meth^-$ (n=5)	7.94± 0.6	7.0 ± 1.2	18.2 ± 1.8	16.2 ± 1.7
Pro^- (n=5)	4.26± 1.4	4.07± 1.4	8.71 ± 2.9	8.4 ± 2.9

ANOVA indicates a treatment affect significant at the $p<0.001$ (see Appendix). Test of least significant difference indicates all groups are significantly different at the $p<0.05$ level.



(a) 5- & 10-min. incubations of Bound Ribosomes



(b) 5- & 10-min. incubations of Free Ribosomes

FIG. 1 COMPARISON BY DIET OF ^{14}C-LEUCINE INCORPORATION MEAN VALUES FOR RAT LIVER RIBOSOMES

other group. This was followed by the lysine-free groups which fell in between the other groups. The methionine-free group was much lower in its ability to synthesize protein than the lysine-free group. The polyribosomes from the protein-free group displayed the least ability to incorporate the ^{14}C amino acid into protein in the in vitro system. This group has the least difference between the counts for the bound and the free polyribosomes (Tables 6, 7). The individual cpm/mg RNA for both the free and bound polyribosomal fractions for each rat are listed in Appendices E and F.

The high rate of incorporation for the casein-free group was expected since casein is defined as a complete protein (i.e., one containing all eight of the essential amino acids(3)). Cottonseed protein is considered to be a good quality protein, although it is limited in two amino acids, methionine and lysine (15). These limiting amino acids could produce an alteration in the polyribosomes which is responsible for the lower rate of incorporation of the ^{14}C-leucine into protein, in comparison to the casein group. The precise type of alteration has not been identified.

These results are in agreement with the work of von der Decken et al. (83) which indicated that the amino acid incorporating activity of rat liver ribosomes was dependent on the biological value of the dietary protein fed to the animals. They showed a decrease in activity when the diet contained either low-quality proteins or suboptimal quantities of protein as compared with adequate amounts of high-quality proteins.

TABLE 6

COMPARISON BETWEEN FREE AND BOUND POLYRIBOSOMES BY GROUP FOR ^{14}C LEUCINE INCORPORATION (FIVE MINUTE INCUBATION)

Treatment Group	No. of Observations	Mean (CPM/mgRNA)	Standard Deviation	Difference (Bound-Free)
Casein				4446*
Bound	10	26612.	3033	
Free	9	22165.	3031	
Cottonseed				2423
Bound	5	23681.	1604	
Free	5	21258.	2713	
Lys-				600
Bound	4	14904.	3866	
Free	5	13667.	2955	
Met-				947
Bound	5	7936.	562	
Free	5	6989.	1167	
Pro-				185
Bound	5	4256.	1373	
Free	5	4070.	1370	

*Statistically significant at p>0.05.

TABLE 7

COMPARISON BETWEEN FREE AND BOUND POLYRIBOSOMES BY GROUP FOR ^{14}C LEUCINE INCORPORATION (10 MINUTES INCUBATION)

Treatment Group	No. of Observations	Mean*	Standard Deviation	Difference (Bound-Free)
Casein				5619**
Bound	9	52258.	3721	
Free	10	46574.	6727	
Cottonseed				5076
Bound	5	47826.	3629	
Free	5	42750.	7676	
Lys-				3975
Bound	5	29403.	4751	
Free	5	25428.	4183	
Met-				1944
Bound	5	18197.	1807	
Free	5	16252.	1673	
Pro-				279
Bound	5	8714.	2884	
Free	5	8434.	2940	

*Statistically significant at $p<0.05$.

These investigators stated that the functional sites of polypeptide formation were not activated to the same extent by various proteins, and this partially accounted for the differences in protein synthesis.

RNA/Protein Ratio

When a rat is starved (30) or fed a protein-deficient diet (17), there is an immediate and extensive loss of RNA for about 2 days, after which a new and lower plateau is attained. From these experiments and subsequent studies (17, 68, 80) it is concluded that the liver RNA content is determined, at least in part, by changes in rate of RNA breakdown regulated by variations in amino acid supply. Ribosomal RNA constitutes 80 to 90% of the total RNA in liver tissue (32); therefore, it was important to examine this parameter in the polysomal fractions. The RNA/protein ratios found in the literature shows a wide range of variation. Munro et al. (42) reported an RNA/protein ratio of 1.2 for total polysomes isolated from rats fed an optimal diet. Values of 0.76 for free polyribosomes and 0.35 for bound polyribosomes were obtained from male rats fed ad libitum (2). The low values for the ratio of 0.12 were obtained by Omstedt and von der Decken (52). They were using ribosomal preparations from rat skeletal muscle, which by techniques used, could have been contaminated, e.g. with other proteins, accounting for the significantly lower values.

Tables 8 and 9 contain the means and standard deviations for RNA and protein, and RNA/protein ratio. These values are given by groups

TABLE 8

AMONG GROUP COMPARISON OF RNA AND PROTEIN CONTENT
OF FREE POLYRIBOSOMAL PELLET

Group	Animals /Groups	Mean * (mg/Pellet)	Standard Deviation	Ratio RNA/Pro
Casein	10			0.74
RNA		1.93	0.043	
Protein		2.59	0.152	
Cottonseed	5			0.73
RNA		1.78	0.072	
Protein		2.48	0.114	
Lys-	5			0.61
RNA		1.37	0.035	
Protein		2.24	0.114	
Met-	5			0.57
RNA		1.15	0.039	
Protein		2.02	0.216	
Pro-	5			0.530
RNA		1.128	0.051	
Protein		2.120	0.164	

*Individual values listed in Appendix K and L.

TABLE 9

AMONG GROUP COMPARISON OF RNA AND PROTEIN CONTENT
OF BOUND POLYRIBOSOMAL PELLET

Group	Animals /Groups	Mean * (mg/Pellet)	Standard Deviation	Ratio RNA/Pro
Casein	10			0.64
RNA		2.93	2.84	
Protein		4.57	4.20	
Cottonseed	5			0.63
RNA		2.76	2.56	
Protein		4.32	4.20	
Lys-	5			0.56
RNA		2.27	2.22	
Protein		4.04	3.90	
Met-	5			0.52
RNA		1.83	1.80	
Protein		3.48	3.40	
Pro-	5			0.51
RNA		1.69	1.66	
Protein		3.28	3.20	

*Individual values listed in Appendix K and L.

for both the bound and free polyribosomal preparations. The casein and cottonseed groups have the highest RNA/protein ratios, and were followed, in descending order, by the lysine-free, methionine-free and protein-free groups. An analysis of variance was run for these three determinations. A significant effect due to the treatment was found, but there was no effect due to day or treatment cross days. This is shown in Tables 10-15. The test of least significant difference was applied to the new data to determine which, if any, groups were different from the control. For the RNA value, all the means were statistically significant except for those between the methionine-free group and the protein-free group for the free polysomes. For the protein values of the bound fractions, all the means were significantly different; whereas, in the free fractions, the casein group was not different from the cottonseed group. There was also no difference found between the methionine-free and protein-free groups or the lysine-free and protein-free groups.

The RNA/protein ratios showed no significant difference between casein and cottonseed, lysine-free and methionine-free, and methionine-free and protein-free. For the bound fraction, there was no difference between the casein and cottonseed groups and the methionine-free and protein-free group. All the other comparisons were significantly different. These results indicate that the RNA content, hence RNA/protein ration, was lowered more severely by the deficient diets, but that there was also, at the same time, a slight decrease in the protein content of the pellet.

TABLE 10

ANALYSIS OF VARIANCE ON RNA FOR BOUND POLYRIBOSOMES

Source of Variation	Degrees of Freedom (n-1)	Sum of Squares	F Value	Probability of Greater F
Treatment	4	7.68	117.5	.001
Day	4	0.02	2.30	.192
Treatment x Day *	16	0.10	2.24	0.189
Error	5	0.01	--	--
Corrected Total	29	7.82	--	--

*Indicates interaction between treatment and day.

TABLE 11

ANALYSIS OF VARIANCE ON RNA FOR FREE POLYRIBOSOMES

Source of Variation	Degrees of Freedom (n-1)	Sum of Squares	F Value	Probability of Greater F
Treatment	4	3.57	405.3	0.001
Day	4	0.01	1.44	0.345
Treatment x Day *	16	0.35	0.99	0.557
Error	5	0.01	--	--
Corrected Total	29	3.63	--	--

*Indicates interaction between treatment and day.

TABLE 12

ANALYSIS OF VARIANCE ON PROTEIN FOR BOUND POLYRIBOSOMES

Source of Variation	Degrees of Freedom (n-1)	Sum of Squares	F Value	Probability of Greater F
Treatment	4	7.65	120.89	.001
Day	4	0.04	0.24	0.905
Treatment x Day *	16	0.25	0.31	0.966
Error	5	0.25	--	--
Corrected Total	29	8.21	--	--

*Indicates interaction between treatment and day.

TABLE 13

ANALYSIS OF VARIANCE FOR PROTEIN FOR FREE POLYRIBOSOMES

Source of Variation	Degrees of Freedom (n-1)	Sum of Squares	F Value	Probability of Greater F
Treatment	4	1.47	19.88	0.001
Day	4	0.13	0.98	0.495
Treatment x Day*	16	0.29	0.53	0.846
Error	5	0.17	--	--
Corrected Total	29	2.08	--	--

*Indicates interaction between treatment and day.

TABLE 14

ANALYSIS OF VARIANCE ON RNA/PROTEIN RATIOS FOR BOUND POLYRIBOSOMES

Source of Variation	Degrees of Freedom (n-1)	Sum of Squares	F Value	Probability of Greater F
Treatment	4	0.09	27.5	0.001
Day	4	0.01	0.3	0.863
Treatment x Day*	16	0.01	0.84	0.640
Error	5	0.01	--	--
Corrected Total	29	0.11	--	--

*Indicates interaction between treatment and day.

TABLE 15

ANALYSIS OF VARIANCE ON RNA/PROTEIN RATIOS FOR FREE POLYRIBOSOMES

Source of Variation	Degrees of Freedom (n-1)	Sum of Squares	F Value	Probability of Greater F
Treatment	4	0.22	44.5	0.001
Day	4	0.17	1.47	0.335
Treatment x Day*	16	0.02	0.44	0.906
Error	5	0.01	--	--
Corrected Total	29	0.28	--	--

*Indicates interaction between treatment and day.

If the data are looked at on a per unit of ribosomes basis, one of two factors could account for the change in the RNA/protein ratio: (a) decrease in the percent of RNA or (b) an increase in the percent of protein. The former is unlikely since a single ribosome contains a fixed number of RNA molecules. Eukaryotic cells contain four RNA molecules/ribosome; therefore, any change must occur in the protein component of the ribosome. If the recriprocal of the RNA/protein is examined, it is seen that the amount of protein per unit RNA increases in the deficient diets. The reason for this increase is only speculative; however, it is possible that this is a compensatory mechanism by which there develops a higher affinity between proteins and the ribosomal unit.

Sucrose Gradient Analysis

Preliminary sucrose gradient analyses were run in order to assure proper technique in the polyribosomal isolation procedure. In addition, analyses were run throughout the experimental procedure as one measure of the effect of feeding the experimental diets vs control diets.

During the experimental procedures, equal portions of each polyribosomal pellet were subjected to analysis to determine if any decreases in the *in vitro* incorporation of ^{14}C leucine into protein synthesis were not due to a lack of polysomes vs monosomes or disomes. Polyribosomes of lower molecular weights have been shown to be less able to synthesize protein when compared to larger polysomes (37). Thus, profiles which indicate

a disproportionate distribution of the monosomes or disomes could account for some loss of ability of the samples to perform *in vitro* protein synthesis. In all pellet samples analyzed, the overall size distribution of ribosomes was in the range of normal expected profiles as obtained during technique development and verification using normal rats and also corresponded to normal profiles found in the literature (60, 78).

The special role of dietary amino acids in controlling the stability of hepatic ribosomes has been extensively documented (14, 18, 45, 52). Studies by Wunner *et al.* (88) suggest that amino acid supply regulates the equilibrium between polysomes, ribosomes and subunits. Inadequate supply of amino acids to liver cells is believed to lead to an increase in free ribosomes with loss of ribosomal RNA from the pool of free ribosomes (30). Associated with the elevation in free ribosomes is the breakdown of the rough endoplasmic reticulum (32). In the present study all of the above factors were also observed. The ability of the ribosomes from the different groups to synthesize protein in a cell-free system showed a direct correlation with the quality of the protein source (Fig. 2 and 3). The mechanisms responsible for this alteration in protein synthesis is only speculative based on the results obtained from the measurements of RNA/protein ratios and on the profiles obtained from the sucrose gradients. Two general mechanisms could operate to alter the protein-synthesizing activity of the ribosomes: one, controlling the proportion of ribosomes that are associated with mRNA and hence are

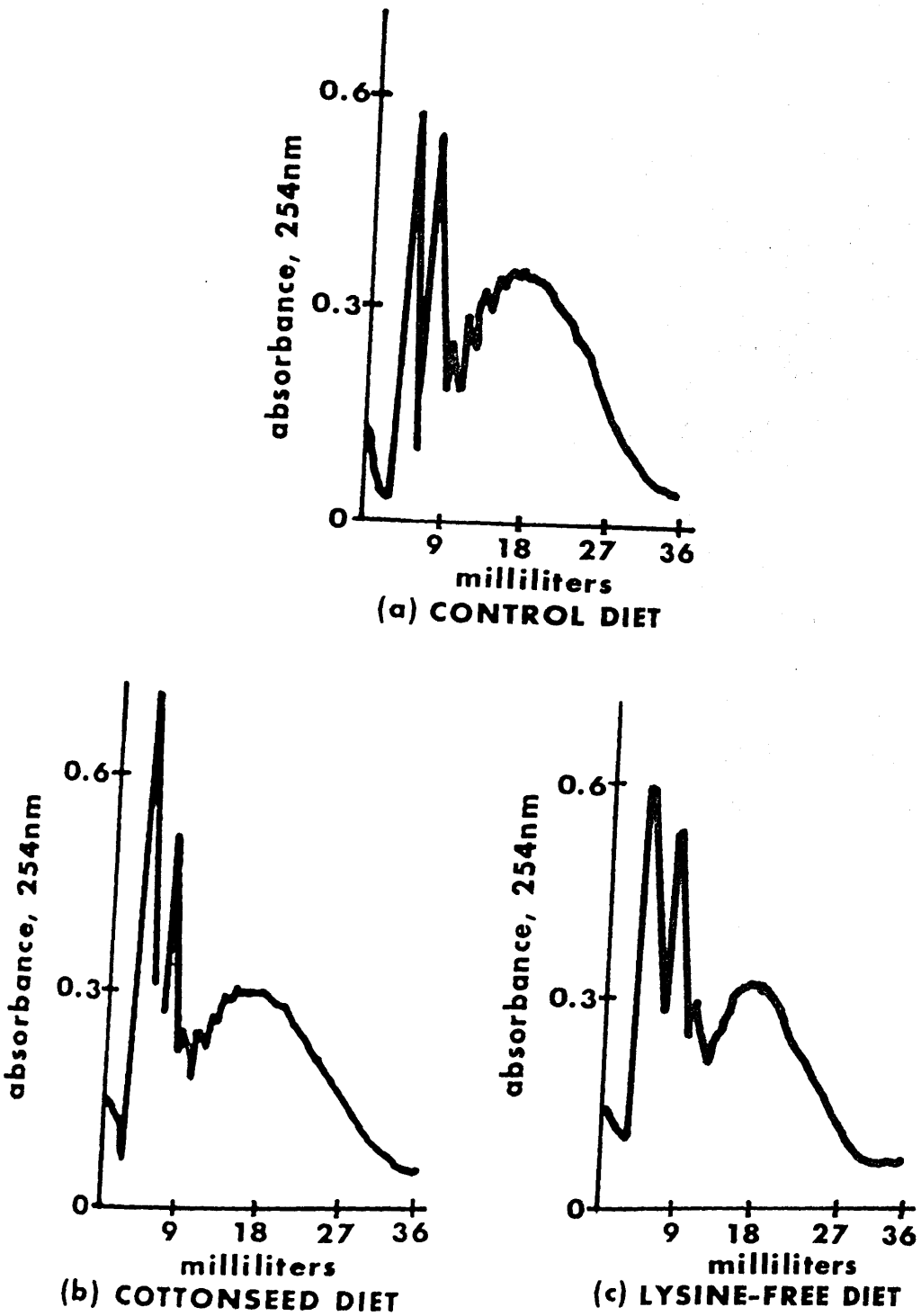
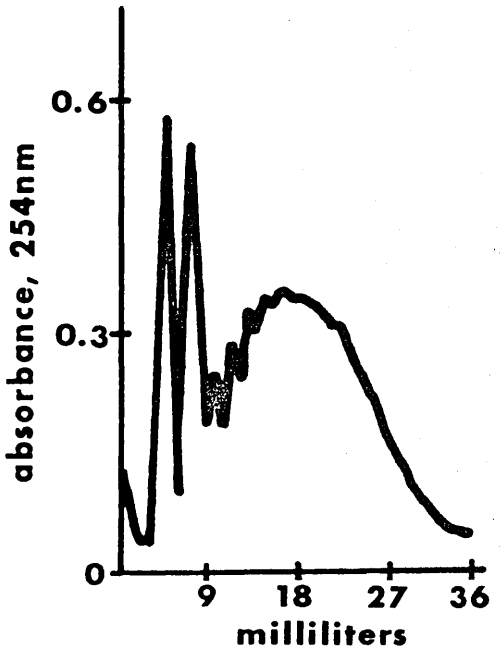
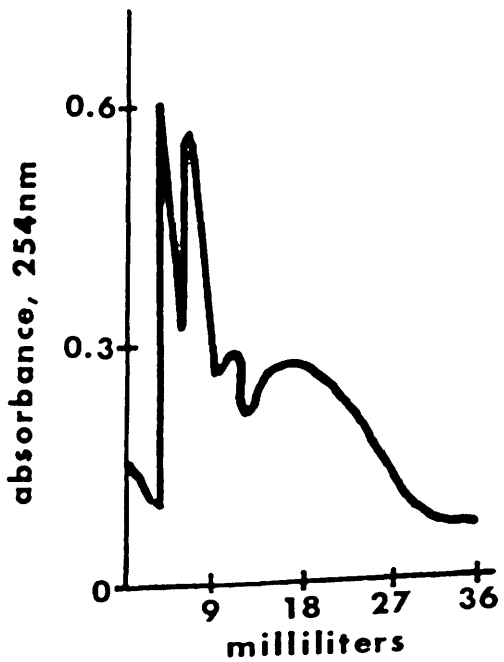


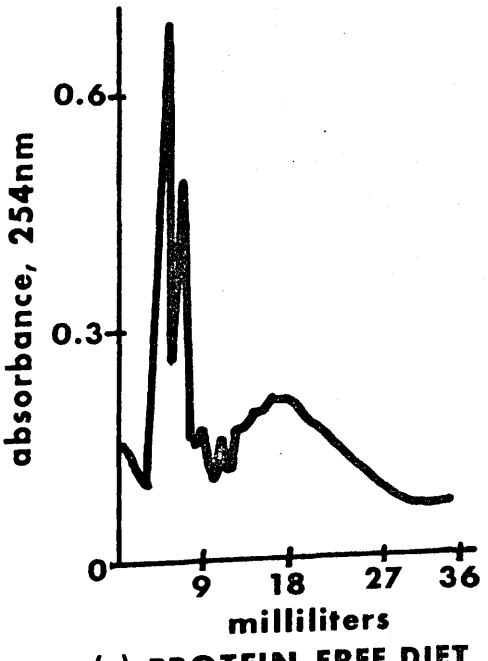
FIG. 2 REPRESENTATIVE SUCROSE GRADIENT ANALYSIS ON CONTROL, COTTONSEED DIET & LYSINE-FREE DIET POLYRIBOSOMAL PREPARATIONS



(a) CONTROL DIET



(b) METHIONINE-FREE DIET



(c) PROTEIN-FREE DIET

FIG. 3 REPRESENTATIVE SUCROSE GRADIENT ANALYSIS ON CONTROL, METHIONINE-FREE DIET, PROTEIN-FREE DIET POLYRIBOSOMAL PREPARATIONS

active in synthesis; the other, regulating the synthesizing activity of the mRNA-associated (polyribosomal) ribosomes. The given data for the amino acid incorporation is expressed in cpm/mg RNA. Therefore, it could be assumed that if all the polysomes are equivalent in structure then they should all have the same specific activity.

The majority of the polysomal peaks from the control group analyses were in the region containing larger polysomes. Similar profiles are found in the literature (60) for normal animals. Profiles from the cottonseed group were similar to those of the casein (control) group with very little apparent alteration in size distribution.

There appeared to be a breakdown of polysomes in rats maintained on the diets lacking in either lysine (Fig. 2) or methionine or protein (Fig. 3). The apparent decreases, seen in the heavy-polysome regions after the various treatments, were accompanied by the appearance of a higher monomer and dimer peak.

The sucrose gradient analyses of the ribosome preparation from the rats fed the experimental diets showed an increase in the proportion of monomeric ribosomes when compared to the control group, and a corresponding fall in the proportion of polyribosomes which could account for some of the decrease in the rate of protein synthesis per mg of RNA. However, this fall appears insufficient to account for all of the large decreases seen. Thus, there could be a depression in polyribosomal activity as well as in the proportion of large polyribosomes.

Since the pool of monomeric ribosomes is replenished through runoff of ribosomes from polyribosomes and is depleted by reassociation of ribosomes with mRNA, the ratio of monomers to polyribosomes reflects the relationship between polyribosome activity and the factors mediating the reassociation of ribosomes with mRNA. The latter factors may be involved in the initiation of new polypeptide chains. Initiation of synthesis of new polypeptide chains occurs only minimally in the liver-cell-free system, so these experiments measure primarily the rate of elongation of previously existing nascent chains (30), that is polyribosome activity.

The alteration in polysomal activity could be brought about primarily through the mechanisms affecting the rate of peptide-bond formation which might occur through alterations in the ribosomes themselves. This alteration could be due to defects in the protein components.

Ancillary Experiment

In order to determine the degree of reliability and reproducibility of this study, a short ancillary experiment was conducted. Five Sprague-Dawley male weanling rats were used. Rats were of a younger age than those used in the primary study because of the lack of availability of male rats of a similar age. Each rat was placed on a 3-day equilibration diet and then fed one of the experimental diets for a 6-day feeding period. On the day following the feeding period, all five rats were sacrificed and the polysomal pellets were prepared. All experimental

procedures were exactly as described in the Methods (Chapter III).

Although the data obtained from this experiment were not identical to those obtained from the primary study, identical values were not expected. The data, however, were in relative agreement to the comparison among groups for all values. In general, there was a lower rate of amino acid incorporation for all groups. The reason for this corresponding, but lower, rate of incorporation could be due to various factors: (a) the age and/or size difference of the rats/ (b) the greater growth rate seen in animals of this age; (c) or normal day-to-day variations in techniques.

Because of the close correlation between the two experiments, it can be stated that the results obtained in the primary study are valid and reproducible under the experimental conditions and procedures used. Specific data on the ^{14}C-leucine incorporation, RNA, protein and RNA/protein ratio are found in Appendix M.

CHAPTER V

SUMMARY AND CONCLUSIONS

Summary

Generally, the purpose of this study was to obtain additional specific data on the nutritional quality of cottonseed flour as an alternate source of dietary protein. Specifically, the study was designed to evaluate whether the feeding of cottonseed flour, as a sole source of nitrogen, produced alterations in the ability of rat liver polysomes to incorporate ^{14}C-leucine into protein.

In order to accomplish the specific purpose, purified rat liver ribosomal pellets were tested for three major parameters: *in vitro* amino acid incorporation ability; ribosomal RNA/protein ratios; and ribosomal size distribution by sucrose gradient analysis.

The ribosomal preparations were prepared from the rat livers of animals fed either a casein diet, which served as a positive control; a protein-free diet which served as a negative control; cottonseed flour which is known to be deficient in lysine and methionine; and a methionine-free or lysine-free diet both which possibly would provide specific comparative criteria by which to further evaluate the cottonseed flour diet.

In Vitro Amino Acid Incorporation

The ability of the ribosomal pellets to incorporate ^{14}C-leucine in an *in vitro* protein synthesis (see Table 4) whether bound or free ribosomes, 5- or 10-min incubations, indicated that the cottonseed-fed animals had a significant depressed incorporation when compared to the positive control. Of the two groups fed the specific amino acid deficient diets (lysine or methionine) the methionine-free samples showed the least ability to incorporate liver leucine. Indications from the data suggest that methionine deficiency may either be a greater limiting factor in protein synthesis or that the deficiency is more pronounced in effect than lysine deficiency in a short-term feeding study. Regardless, data does indicate that the cottonseed diet, when fed to this animal model for this time frame, does by the procedure of Ramsey and Steele (60) inhibit slightly the incorporation of ^{14}C-leucine by the rat liver polyribosomes. The decrease in incorporation although slight, is statistically significant when analyzed by the test as outlined in the Methods.

RNA/Protein Ratio

To further examine the mechanism of this deficiency, the RNA/protein ratio of each ribosomal pellet was determined in duplicate. Ribosomes are reported to contain approximately equal amounts of RNA and protein. Therefore, one would expect to find an RNA/protein ratio of approximately 1.0 for ribosomes isolated from the livers of rats fed a

nutritionally adequate diet. The actual ratio values were lower than expected, but for example, the bound polysomes (Table 12) showed a decrease which paralleled the decrease seen in the incorporation ability (Table 4). The same slight between-group differences were also seen for the free polysomal ratios (Table 11). Although a longer feeding period may have shown a more pronounced effect, the rationale for obtaining the RNA/protein ratio of the polysomes was to determine whether a lack of RNA or protein specifically could account for corresponding incorporation ability. Based on this experimental design, the animal model selected, and length of feeding time, a definitive statement would be inappropriate. However, the RNA/protein ratio decrease and corresponding decline in the rate of amino acid incorporation, strongly suggest that a reduction in the quantity of ribosomes could be partially responsible.

Sucrose Gradient Analysis

Prior to initiation of the actual experiment, sucrose gradient analyses were conducted during the technique development stage of isolating the polyribosomal pellets. This preliminary data indicated that the preparation of the polyribosomes provided pellets with an adequate distribution between lower molecular weight (monosomes and disomes) and the higher weight molecules (polysomes) to ensure that any alteration in the protein-synthesizing activity would be due to causes other than technique. Sucrose gradient analyses were continued throughout the experimental period to maintain consistency. Representative examples of

these gradients are shown in Fig. 2 and 3.

The sucrose gradient analysis of the ribosomal preparation from the rats fed the experimental diets showed an increase in the proportion of monomeric ribosomes with a corresponding decrease in polysomes when compared to the control group. This fall in the proportion of polysomes may account for some of the decrease in the rate of protein synthesis per mg of RNA. However, this fall seems insufficient to account for the total decrease in protein synthesis that was observed. Thus, a depression in polysomal activity could be concluded as the cause of this difference.

Conclusions

From the results of this experiment, certain conclusions regarding the ability of rat liver polysomes to synthesize protein *in vitro* can be drawn.

1. The cottonseed group had only a slight reduction in amino acid incorporation when compared with the casein control group.
2. The cottonseed group had a much higher rate of incorporation than did the methionine-, lysine- or protein-free groups.
3. There was a reduction in the RNA/protein ratio which paralled the decrease in the amino acid incorporation.

4. The sucrose gradient analyses showed that there was an alteration in the size distribution of polysomes found in the deficient diets. There was a decrease in the amount of large polysomes and an increase in monosomes.

The mechanisms responsible for the decrease in protein synthesis could be due to one or both of two primary factors: (a) a decrease in the size and amount of the polyribosomes and/or (b) a decrease in the activity of the ribosomes. In this investigator's opinion, as evidenced by the data obtained experimentally and information contained in the literature, a combination of both factors plays a significant role in altering the protein synthesizing activity of the polyribosomes. Neither factor, considered separately, could account for the degree of decrease in activity noted for the animals on deficient diets.

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APPENDIX

APPENDIX A

COMPARISON OF AMINO ACID COMPOSITION OF CASIN, GLANDLESS COTTONSEED FLOUR, AND OVALBUMIN

Protein	Amino Acid Content (g/100 g of diet *)						
	Meth	Cys	Total S-AA	Tryp	Lys	Phe	Thr
Casein	0.492	0.060	0.552	0.179	0.939	0.581	0.596
Cottonseed	0.243	0.416	0.659	0.209**	0.680	0.956	0.516
Ovalbumin	0.358	0.268	0.626	0.194	0.760	0.760	-
RDA for rat (g/100 g of diet)	0.600	-	0.600	0.200	1.000	0.700	0.500

*15 percent protein by weight.
**Approximate value.

APPENDIX B

COMPOSITION OF SALT MIXTURE R. H.

Ingredient	Percentage
Ammonium molybdate·$4H_2O$	0.003
Calcium carbonate	29.290
Calcium phosphate	0.430
Cupric sulfate	0.156
Ferric citrate·$6H_2O$	0.623
Magnesium sulfate·$7H_2O$	9.980
Manganese sulfate·H_2O	0.121
Potassium iodide	0.001
Potassium phosphate	34.310
Sodium chloride	25.060
Sodium selenite·$5H_2O$	0.002
Zinc chloride	0.020

The salts at 5 percent of the diet provided in percent of element: Ca, 0.592; P, 0.394; K, 0.493; Na, 0.493; Cl, 0.760; Mg, 0.049; Fe, 0.0049; Cu, 0.0019; Mn, 0.00195; Zn, 0.0004; I, 0.000019; Mo, 0.000005; Se, 0.0000024 (64).

APPENDIX C

COMPOSITION OF VITAMIN DIET FORTIFICATION MIXTURE

Ingredient	mg/100 g. diet
Vitamin A concentrate	2000. (IU)
Vitamin D concentrate	200. (IU)
Alpha-tocopherol	11.000
Ascorbic acid	100.000
Inositol	11.000
Choline chloride	165.000
Menadione	4.900
p-Aminobenzoic acid	11.000
Niacin	9.900
Riboflavin	2.200
Pyridoxine hydrochloride	2.200
Thiamine hydrochloride	2.200
Calcium pantothenate	6.600
Biotin	0.050
Folic acid	0.190
Vitamin B-12	0.003

APPENDIX D

MATERIALS

L-[U-^{14}C] Leucine - Aquenous solution containing 2% ethanol, sterilized 330 mCi/mmol. Batch 123, Amersham/Searle Corporation, Arlington Heights, Illinois.

Scintanalyzed, Scinti Verse. Fisher Scientific Company, Fair Lawn, New Jersey.

HEPES Buffer - (n-2 Hydroxyethylpiperazine N'-2-ethanesulfonic acid) Sigma Chemical Company, St. Louis, Missouri.

KCl - Potassium chloride - Reagent grade - Fisher Scientific Company, Fair Lawn, New Jersey.

KOH - Potassium Hydroxide - Reagent Grade - Fisher Scientific Company, Fair Lawn, New Jersey

$MgCl_2$ - Magnesium chloride - Reagent Grade - Fisher Scientific Company, Fair Lawn, New Jersey.

EDTA - Disodium Ethylenediamine-tetraacetate - Reagent Grade - Fisher Scientific Company, New Jersey.

L-Leucine - Reagent Grade - Fisher Scientific Company, New Jersey.

TCA - Trichloro-acetic acid - Reagent Grade - Fisher Scientific Company, New Jersey.

DOC - Deoxycholic Acid, sodium salt - Reagent Grade - Sigma Chemical Company, St. Louis, Missouri.

Alpha-Amylase (α-1, 4-Glucan 4-glucanohydrolase; E.C. No. 3.21.1) 1 unit=1.0 mg protein in 3 min.

Phosphocreatine - (creatine phosphate) Disodium salt - Reagent Grade - Sigma Chemical Company, St. Louis, Missouri.

ATP - Adenosine 5'-Triphosphate - Reagent Grade - Sigma Chemical Company, St. Louis, Missouri.

GTP - Guanosine 5'-Triphosphate sodium salt - Reagent Grade - Sigma Chemical Company, St. Louis, Missouri.

APPENDIX D (continued)

Creatine Phosphokinase - 163 International Unit mg protein - Sigma Chemical Company, St. Louis, Missouri.

Triton X-100 - Scintillation Grade - Amersham/Searle Corporation, Arlington Heights, Illinois.

Sephadez G-10 - Pharmacia Fine Chemicals Inc., Piscataway, New Jersey.

Glutathione, reduced - ICN Pharmaceuticals, Inc., Plainview, New York.

Sucrose - Density Gradient Grade (Ribonuclease Free). Schwarz/Mann, Orangeburg, New York.

Carbon Declorizing Neutral, Norit. - Fisher Scientific Company, Fair Lawn, New Jersey.

EXPERIMENTAL AND CONTROL DIETS

Casein diet (Control diet) - The vitamin free casein (contains 91.4 per cent protein) and vitamin free fortification mixture were obtained from Nutritional Biochemicals Co., Cleveland, Ohio. Casein diet was supplemented with 0.3 per cent L-methionine.

Glandless Cottonseed flour diet - The cottonseed flour diet (contains 57 percent protein) was obtained from Texas A & M University, College Station, Texas.

Synthetic diet - The amino acid defined diet with methionine omitted or lysine omitted (Teklad Mills Division of the Mogul Co., Chagrin Falls, Ohio) contained 14.9 per cent protein according to Micro-Kjeldahl analysis. When amino acids were omitted from the diets, they were replaced by an equivalent amount of glysine so that the diets were isonitrogenous. All amino acids used were L-isomers. The composition of the complete synthetic amino acid diet is shown in Appendix A.

APPENDIX E

COMPARISON OF BOUND AND FREE POLYRIBOSOMAL COUNTS BY ANIMAL PER DAY (5 Min. Incubation)

Treatment Group	Animal No.	Day of Treatment	Polyribosomes* Bound	Free
Casein	1	1	29060.1	17139.2
	2	1	27186.8	21926.1
	1	2	31736.2	18987.1
	2	2	28780.5	22123.7
	1	3	21693.2	26097.8
	2	3	22223.1	25354.0
	1	4	27111.1	23967.4
	2	4	25661.0	25405.1
	1	5	26965.5	20639.8
	2	5	25704.2	20015.4
Cottonseed	3	1	25378.5	21021.7
	3	2	24014.0	22462.3
	3	3	21020.1	25225.4
	3	4	24091.4	18938.8
	3	5	23905.9	18643.5

*cpm/mgrRNA

APPENDIX E (continued)

COMPARISON OF BOUND AND FREE POLYRIBOSOMAL COUNTS
BY ANIMAL PER DAY
(5 Min. Incubation)

Treatment Group	Animal No.	Day of Treatment	Polyribosomes* Bound	Free
Lys-	4	1	19354.8	13873.2
	4	2	15182.8	10459.9
	4	3	9913.7	15273.0
	4	4	15167.8	17610.1
	4	5		11122.2
Meth-	5	1	7884.4	5706.3
	5	2	8142.0	6330.7
	5	3	7013.3	8698.6
	5	4	8133.2	7565.8
	5	5	8509.7	6644.4
Pro-	6	1	5629.7	5918.0
	6	2	5793.6	4960.8
	6	3	2813.4	3763.1
	6	4	3259.4	3215.3
	6	5	3786.8	2496.0

*cpm/mgrRNA

APPENDIX F

COMPARISON OF BOUND AND FREE POLYRIBOSOMAL COUNTS BY ANIMAL PER DAY (10 Min. Incubation)

Treatment Group	Animal No.	Day of Treatment	Polyribosomes* Bound	Free
Casein	1	1	55030.1	31752.0
	2	1	.	45996.7
	1	2	56931.3	39478.8
	2	2	55890.8	46594.9
	1	3	47420.3	54863.7
	2	3	49013.5	53718.9
	1	4	55350.9	47396.1
	2	4	52618.2	49551.0
	1	5	47681.9	47088.7
	2	5	50388.0	49302.7
Cottonseed	3	1	47890.8	40714.8
	3	2	53438.9	48754.8
	3	3	44338.5	52678.9
	3	4	48554.3	36233.2
	3	5	44912.2	35371.6

*cpm/mgrRNA

APPENDIX F (continued)

COMPARISON OF BOUND AND FREE POLYRIBOSOMAL COUNTS
BY ANIMAL PER DAY
(10 Min. Incubation)

Treatment Group	Animal No.	Day of Treatment	Polyribosomes* Bound	Free
Lys-	4	1	34582.9	26085.7
	4	2	32476.3	23715.3
	4	3	22630.0	30515.7
	4	4	30541.9	27451.8
	4	5	26786.2	19373.1
Met-	5	1	17534.4	16191.1
	5	2	17742.0	15258.0
	5	3	15814.7	19107.3
	5	4	20500.9	14882.0
	5	5	19394.8	15825.9
Pro-	6	1	13639.0	12299.9
	6	2	8758.9	10665.3
	6	3	6851.9	7586.6
	6	4	6556.2	6119.4
	6	5	7768.4	5503.2

*cpm/mgrRNA

APPENDIX G

ANALYSIS OF VARIANCE AMONG GROUPS FOR ^{14}C LEUCINE INCORPORATION OF FREE POLYRIBOSOMES
(Incubated 5 Min.)

Source of Variation	Degrees of Freedom (n-1)	Sum of Squares	Expected Mean Square	F Value Ratio	Probability >F
Treatment	4	3087689785.	$\sigma^2+c_1\sigma^2_{\alpha\beta}+c_2 \sum_{i=1}^{5}\alpha_i^2$	86	.001
Day*	4	110684488.	$\sigma^2+c_3\sigma^2\beta$	9	.001
Treatment x Day	16	142203886.	$\sigma^2+c_3\sigma^2\alpha\beta$	3	.003
Error	31	90049060.	σ^2	--	--
Corrected Total	55	3430627221.	--	--	--

*Refers to day of sacrifice

APPENDIX H

ANALYSIS OF VARIANCE AMONG GROUPS FOR ^{14}C LEUCINE INCORPORATION OF FREE POLYRIBOSOMES
(Incubated 10 Min.)

Source of Variation	Degrees of Freedom (n-1)	Sum of Squares	Expected Mean Square	F Value Ratio	Probability >F
Treatment	4	13809006806.	$\sigma^2+c_1\sigma^2_{\alpha\beta}+c_2\sum_{i=1}^{5}\alpha_i^2$	71	.001
Day*	4	475644308.	$\sigma^2+c_3\sigma^2\beta$	10	.001
Treatment x Day*	16	777706702.	$\sigma^2+c_1\sigma^2\alpha\beta$	4	.002
Error	35	410013872.	σ^2	--	--
Corrected Total	59	15472371689.	--	--	--

*Refers to day of sacrifice

APPENDIX I

ANALYSIS OF VARIANCE AMONG GROUPS FOR ^{14}C LEUCINE INCORPORATION OF BOUND POLYRIBOSOMES
(Incubated 5 Min.)

Source of Variation	Degrees of Freedom (n-1)	Sum of Squares	Expected Mean Square	F Value Ratio	Probability >F
Treatment	4	4764772796.	$\sigma^2+c_1\sigma^2_{\alpha\beta}+c_{2i}\sum_{=1}^{5}\alpha_i^2$	210	.001
Day*	4	192339743.	$\sigma^2+c_3\sigma^2_{\beta}$	18	.001
Treatment x Day*	15	85052650.	$\sigma^2+c_1\sigma^2_{\alpha\beta}$	2	.020
Error	34	87333404.	σ^2	--	--
Corrected Total	57	5129498594.	--	--	--

*Refers to day of sacrifice

APPENDIX J

ANALYSIS OF VARIANCE AMONG GROUPS FOR ^{14}C LEUCINE INCORPORATION OF BOUND POLYRIBOSOMES
(Incubated 10 Min.)

Source of Variation	Degrees of Freedom (n-1)	Sum of Squares	Expected Mean Square	F Value Ratio	Probability >F
Treatment	4	15624846099.	$\sigma^2+c_1\sigma^2_{\alpha\beta}+c_2\sum_{i=1}^{5}\alpha_i^2$	372	.001
Day*	4	388492370.	$\sigma^2+c_3\sigma^2\beta$	20	.001
Treatment x Day*	16	167914295.	$\sigma^2+c_1\sigma^2\alpha\beta$	2	.031
Error	29	138247737.	σ^2	--	--
Corrected Total	53	16319400502.	--	--	--

*Refers to day of sacrifice

APPENDIX K

COMPARISON OF RNA, PROTEIN AND RNA/PROTEIN RATIOS BY ANIMAL PER DAY OF SACRIFICE (Free Polyribosomes)

Treatment Group	Day	RNA	Protein	RNA/Protein
Casein	1	1.97	2.60	0.75
		1.96	2.70	0.72
	2	1.94	2.80	0.69
		1.95	2.50	0.78
	3	1.91	2.60	0.73
		1.97	2.60	0.75
	4	1.94	2.40	0.80
		1.86	2.70	0.68
	5	1.86	2.30	0.80
		1.97	2.70	0.72
Cottonseed	1	1.91	2.40	0.79
	2	1.75	2.50	0.70
	3	1.78	2.30	0.77
	4	1.72	2.40	0.71
	5	1.78	2.60	0.68
Lys$^-$	1	1.43	2.20	0.65
	2	1.37	2.40	0.57
	3	1.38	2.10	0.61
	4	1.35	2.20	0.61
	5	1.34	2.30	0.58
Met$^-$	1	1.13	1.70	0.66
	2	1.16	2.10	0.55
	3	1.22	1.90	0.64
	4	1.15	2.20	0.52
	5	1.12	2.20	0.50
Pro$^-$	1	1.11	2.00	0.55
	2	1.10	2.10	0.52
	3	1.10	2.00	0.50
	4	1.11	2.10	0.52
	5	1.22	2.40	0.51

APPENDIX L

COMPARISON OF RNA, PROTEIN AND RNA/PROTEIN RATIOS BY ANIMAL PER DAY OF SACRIFICE (Bound Polyribosomes)

Treatment Group	Day	RNA	Protein	RNA/Protein Ratio
Casein	1	3.03	4.40	0.68
		2.95	4.80	0.61
	2	2.84	4.60	0.61
		2.93	4.90	0.59
	3	2.87	4.40	0.65
		2.95	4.80	0.61
	4	2.93	4.50	0.65
		3.01	4.60	0.65
	5	2.91	4.20	0.69
		2.89	4.50	0.64
Cottonseed	1	2.68	4.40	0.60
	2	2.89	4.30	0.67
	3	2.93	4.30	0.68
	4	2.75	4.20	0.65
	5	2.56	4.40	0.58
Lys^-	1	2.25	4.00	0.56
	2	2.33	4.20	0.55
	3	2.22	3.90	0.56
	4	2.31	4.00	0.57
	5	2.24	4.10	0.54
Met^-	1	1.83	3.40	0.53
	2	1.81	3.40	0.53
	3	1.87	3.50	0.53
	4	1.83	3.50	0.52
	5	1.80	3.60	0.50
Pro^-	1	1.73	3.20	0.54
	2	1.69	3.30	0.51
	3	1.66	3.40	0.48
	4	1.72	3.30	0.52
	5	1.67	3.20	0.52

APPENDIX M

AMINO ACID INCORPORATION OF ^{14}C LEUCINE BY RAT LIVER POLYRIBOSOMES: ANCILLARY EXPERIMENT

Group	5-Min. Incubation*		10-Min. Incubation*	
	Free	Bound	Free	Bound
Casein	24686	25509	47925	52659
CS**	20560	24747	39029	48578
Lys^-	16489	18141	34985	33325
Met^-	7724	9180	15237	17507
Pro^-	4650	4995	7987	8858

*Values listed are an average of duplicate samples.
**Glandless Cottonseed Flour.