THE EFFECTS OF AN AMINO ACID DEFICIENT WHEAT GLUTEN DIET UPON PROTEIN BIOSYNTHESIS IN RATS

by

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ABSTRACT

Protein-calorie malnutrition is one of the major world health problems. The consumption of cereal proteins, commonly deficient in one or more essential amino acids, results in a series of morphological and biochemical changes. In-vivo studies have indicated that there is increased protein biosynthesis in the livers of animals consuming diets containing poor quality proteins. This dissertation has examined the mechanism of this enhancement.

Eight groups of Sprague-Dawley weanling female rats, eight test and four control rats per group, were fed diets equal in all respects except protein quality for 33 days. Wheat gluten, a low quality protein, comprised 20 per cent of the test diet which was replaced by casein, a high quality protein, in the control diet. Food and water were supplied ad libitum. All animals were fasted 17 hours before sacrificing. Test animals consumed less food, grew less and had lower food efficiency ratios than the controls.

Cell sap and microsomes from the livers of rats fed wheat gluten incorporated 158 per cent more leucine-C^{14} than the controls. Crossing the fractions indicated that the microsomes were primarily responsible for the increment. The crude protein synthesizing fractions were partially purified in an effort to elucidate the mechanism of increased activity. p^H 5 enzymes and RNP particles from test livers also incorporated more leucine-C^{14} than

the controls. Regardless of the enzyme concentration, the test system incorporated more leucine-C^{14}. When activating and transfer enzymes (1 mg) eluted from a DEAE-cellulose column, phenol extracted s-RNA (0.2 mg) and RNP particles (1 mg RNA) were incubated, the specific activity of the test system was 42 per cent greater than the control. Incubations containing labelled phenylalanine, lysine and alanine gave similar results. Also, the relative incorporation was the same in the tests and controls. Therefore it is concluded that the increment is a general phenomenon. It also suggests that the liver particles from the two dietary groups may be synthesizing similar polypeptides. Adding free amino acids to the incubation mixtures increased the specific activity of both systems equally.

In the purified system, both the ribosomal and enzyme fractions contributed to the increased activity. Incubating increasing amounts of enzyme and s-RNA with a constant amount of RNP-RNA indicated that the maximum velocity of the wheat gluten group was greater. This, with the same Michaelis-Menten (K_m) constant for the two groups suggests an irreversible type of stimulation by enzymes and ribosomes from the livers of rats fed wheat gluten.

Except for a slightly larger monomer peak in the particles from the rats fed wheat gluten (which might be attributed to the increased concentration of metallo-proteins), there was no change in the sedimentation pattern of the RNP particles. Therefore it is concluded that the difference in the activity of the ribosomal fraction is at least in part an intrinsic property of the RNP

particles themselves. It appears that the difference in the activity of the enzyme fraction may be due to a change in the transfer enzymes.

INTRODUCTION

Malnutrition is one of the major problems in the world today. More than one-half of its inhabitants are hungry or malnourished (F.A.O., 1964). Since cereals are usually the principal component of the diet in areas where malnutrition prevails, protein is the most common limiting metabolite. It is the rapidly growing pre-school child who suffers most from this deficit.

Czerny and Keller (1906) were the first to describe protein malnutrition, Mehlnährschaden, in children. Correa (1908) described the same syndrome in Yucatán--Culebrilla. By 1952, it was well-established that protein malnutrition was wide-spread (Waterlow, Cravioto and Stephen, 1960). World-wide investigations have continued and protein-calorie malnutrition is now recognized as one of the major health problems. The many reviews covering this subject are too numerous to enumerate here. A more recent one by Viteri et al. (1964) presents the picture admirably and in a well-documented form.

Two Types of Protein-Calorie Malnutrition

It is now generally agreed that protein-calorie malnutrition should be divided into two categories (F.A.O., 1962). In one type, both calorie and protein intakes are limited; in the other, protein intake remains low but

total caloric intake is relatively normal. These two dietary conditions give rise to different biochemical pictures.

In severe cases of malnutrition, the first type manifests itself in a pathological syndrome referred to as "marasmus." Children demonstrate decreased metabolic activity and their cells divide more slowly (Mönckeberg, 1967). The activity of the enzymes involved in urea synthesis decrease (Schimke, 1962) and urinary nitrogen is low. Plasma protein and amino acid concentrations are almost normal, with a much longer half-life for albumin. There is gradual muscle wasting (see Mönckeberg, 1967). These conditions suggest metabolic adaptation to protein-calorie deficiency by reduced metabolic expenditure.

There is also evidence of concomitant changes in hormonal levels. Among these are, decreased somatotropic hormone (Monckeberg et al., 1963); thyroid hypofunction, due to a lack of thyroid stimulating hormone (Beas et al., 1966); increased 17-hydroxycorticosteroid excretion (Castellanos and Arroyave, 1961). The latter hormone exerts a catabolic effect on carcass protein and an anabolic effect on the viscera (Goodlad and Munro, 1959).

The second type of malnutrition, given the name "kwashiorkor" by the first F.A.O./W.H.O. Joint Expert Committee on Nutrition (1952), presents a different biochemical picture. Here, dietary amino acids, rather than calories, are the first limiting metabolite. The body does not need to catabolize muscle or transport fat to fulfill caloric needs which, it appears, take precedence over other physiological requirements (Viteri et al., 1964).

The basal metabolic rate is normal and cellular division is only slightly decreased (see Mönckeberg, 1967). Total urinary nitrogen, urea and creatinine are below average but other nitrogenous fractions, including amino acids and abnormal metabolites, tend to be high (Edozien and Phillips, 1961). It appears that the visceral organs suffer an acute amino acid deficit. Serum amino acid, lipid and albumin concentrations are low. Proteins synthesized in the liver and pancreas decrease, resulting in edema, fatty livers, hemosiderosis, etc. (as reviewed and reported by Viteri et al., 1964). Decreased glucocorticoid hormone activity prevents mobilization of carcass protein reserves for use by the vital organs. "The fact that glucocorticoid excretion is high in marasmus and low in kwashiorkor is of importance and explains many of the differences between these two syndromes" (Arroyave, 1965).

It should be borne in mind, however, that marasmus and kwashiorkor are the extremes of a continuous spectrum in which varying degrees of relative deficiency of protein and calories are found (Viteri et al., 1964). True classical cases are in the minority. A child may exist in a malnourished state for a long time. Suddenly some traumatic condition, i.e., a stress such as infection, may precipitate kwashiorkor symptoms (Scrimshaw, 1962). It is thus possible that the failure to produce glucocorticoid hormones may result in an inability to cope with an added stress in a child who is already in precarious balance. The exact mechanism of decreased glucocorticoid production is not known.

Protein Requirements of Young Children

The exact protein requirements of young children are not known. In 1957, the F.A.O. set up a provisional reference amino acid pattern that was derived from values of human requirements for individual amino acids, when consumed in a mixture of free amino acids. Subsequent pattern revisions have been recommended but many questions still remain and there is a dearth of information on dietary protein requirements of children in various physiological states. It appears that the essential amino acid (EAA) pattern for children is similar to that for adults but children require proportionately more EAA per gram of dietary protein (Food and Nutrition Board, 1963).

The Food and Nutrition Board (1964, p. vii) recommends 32 grams of protein daily for the 1 to 3 year old child. This calculates out to about 10 per cent of the total dietary calories. In view of the higher EAA requirements, it is recommended that 25 to 50 per cent of the protein should be supplied by high quality proteins (see below). The authors contend that "these allowances should provide approximately twice the minimal need for the average child and allow a reasonable margin for the rapidly growing child" (Food and Nutrition Board, 1964, p. 15).

Protein Quality

The nutritional value of a protein depends on the proportion of the various EAA that it contains. Numerous methods including chemical score, biological value, etc., have been devised to evaluate the quality of a protein.

The Food and Nutrition Board of the National Research Council (1963) published an excellent review of protein quality. The more closely the proportions of absorbed EAA correspond with the EAA requirements of the animal (the reference pattern), the more efficiently the protein may be used in the body. Efficient utilization of dietary proteins also depends on the ratio of EAA to non-EAA as well as the per cent of protein in the diet. If the quantity of one of the EAA is low in a particular protein, a large amount of that protein must be ingested and metabolized in order to get a sufficient amount of the deficient amino acid. This results in low efficiency (Food and Nutrition Board, 1963, p. 19).

If the degree of imbalance among EAA is great enough, it may have other untoward effects in the animal. These include an increased requirement for the amino acid in greatest deficit as well as anorexia (Harper, 1964, p. 100). Harper has defined imbalance as "those changes in the amino acid pattern of a diet that cause a growth depression which is completely prevented by a small supplement of the limiting amino acid or acids" (Harper, 1964, p. 129). Cereals are principal constituents of the diets of protein malnourished children. These grains contain approximately 12 per cent protein (Proudfit, 1961) which, in turn, accounts for 13 per cent of the calories. However, this protein is of low quality; lysine, tryptophan, methionine and threonine being among the EAA that are often in short supply.

Effects of Feeding Diets Containing Imbalanced Proteins

Morphological effects

Studies with experimental animals have shed some light on the morphological and biochemical effects of low quality proteins. In designing such studies a significant problem is decreased food consumption (Harper, 1958). Sanahuja and Harper (1962) found that rats on an adequate diet and subsequently transferred to an amino acid imbalanced diet reduced their food consumption within a few hours. When protein-depleted rats were offered a choice of a diet containing imbalanced protein versus a protein-free diet, they showed a preference for the protein-free diet after the third day. All rats, whether protein-depleted, starved, or well-fed, eventually preferred the protein-free diet. Time for this response varied, depending on prior treatment (Sanahuja and Harper, 1963). However, it should be remembered that changing the protein reserves modified the experimental conditions.

In order to avoid the problem of decreased food intake, Sidransky and his associates (Sidransky and Farber, 1958a, 1958b; Sidransky and Baba, 1960; Sidransky and Verney, 1964) force-fed weanling rats diets, devoid of one EAA that contained 17.3 per cent protein (9.2 per cent EAA and 8.1 per cent non-EAA) for 3 to 7 days. The omitted EAA was added to the control diet. From the results obtained by the omission of each of the EAA except tryptophan, they concluded that the pathological consequences of histidine, isoleucine, phenylalanine, threonine, or valine-devoid diets (HIPTV) were essentially the same (Sidransky and Verney, 1964). Many of the changes were also observed

with leucine, lysine or methionine deficiencies. All animals, except those receiving the arginine devoid diet, decreased in body weight. The changes per liver, when compared with livers from control animals, were as follows: the weight of the organ increased in the HIPTV and arginine groups; protein content decreased in the leucine group; lipid content increased in HIPTV, lysine and methionine groups; glycogen content increased in all except the methionine group. Skeletal muscle weight and protein content decreased in all except the arginine groups. The pancreas, spleen, and salivary glands atrophied in all except the methionine and arginine groups.

These authors concluded that, with the exception of the arginine and methionine-devoid diets, they had produced kwashiorkor-like symptoms. They likewise observed that the pathological changes observed in the force-fed animals could be attributed to a metabolic imbalance induced by a variety of factors and not a single deficiency.

The changes were not as evident when the amino acid-devoid diets were fed ad libitum for 7 days. Livers from animals that consumed a threonine- or methionine-devoid diet weighed less; liver protein and lipid content decreased while glycogen content increased. Muscle weight and mg protein per muscle were significantly less. When valine- or lysine-devoid diets were fed (Sidransky and Baba, 1960), values for all of the above-mentioned constituents were less than the control. It appears that this condition more closely resembles marasmus.

Sanahuja, Rio and Lede (1965) created a threonine-deficient diet by supplementing wheat gluten protein (10 per cent of the diet) with lysine and then adding 3.1 per cent of an EAA mixture lacking threonine. Threonine was added to the control diet. These diets were fed ad libitum to weanling rats for 2 weeks. When compared with controls, the liver weight of the threonine-deficient (per 100 grams body weight) increased as did liver glycogen while lipid content decreased. Increased liver glycogen appears to be a common result of feeding amino-acid deficient diets.

Biochemical effects

Banks, Allison, and Wannemacher (1964) compared the effects of feeding wheat gluten, supplemented wheat gluten, casein, and egg albumin proteins. They concluded that nitrogen intake from different sources, fed in quantities sufficient to produce the same weight gain, resulted in different biochemical responses in various tissues. Liver RNA/DNA and protein/DNA ratios were significantly lower in rats fed wheat gluten than they were in animals fed casein. RNA/protein was the same for all diets. The amount of protein in the liver is influenced directly by both quantity and quality of dietary protein (Allison et al., 1964).

In-vivo C^{14}-amino acid incorporation. In an effort to gain more information on how these imbalanced proteins are metabolized, many researchers have utilized radioactive isotopes. Liver protein synthesis is often estimated by measuring the amount of radioactive amino acids incorporated.

Stirpe and Schwarz (1963) fed a 30 per cent torula yeast diet, deficient in methionine, for 10 to 14 days. They injected valine-C^{14} 4 hours before killing and found enhanced valine incorporation into liver proteins when compared to rats on a casein diet. Nimni and Bavetta (1961) reported that when rats, previously fed a 24 per cent acid casein hydrolysate (tryptophan free) diet for 13 days, were injected intramuscularly with glycine-C^{14} and sacrificed 5 hours later, the specific activity of the liver and serum proteins was higher than that of the controls. Garrow (1959) and Waterlow (1959) found similar increases in C^{14}-amino acid incorporation into liver proteins of protein-depleted animals.

Sidransky and Farber (1958b) observed that when rats, force-fed methionine or threonine-devoid diets, were injected intraperitioneally with C^{14}-amino acids, they incorporated more radioactive amino acids into liver and plasma proteins than control animals. The same results were noted, using leucine- or isoleucine-devoid diets (Sidransky and Verney, 1964).

Fasted rats, previously trained to eat twice daily, were fed a histidine-deficient diet (containing histidine-N^{15}) and they incorporated more label into all tissues than control animals which consumed adequate histidine (Hartman and King, 1967). Harper et al. (1964), also found that when a tracer amount of C^{14}-amino acid (threonine or histidine) was given in a single meal, the liver of the animal consuming the imbalanced protein diet took up significantly more label than the control while muscle incorporation was about the same in both groups.

Free amino acid concentration. Harper and his group showed in several experiments (Sanahuja and Harper, 1963; Kumta and Harper, 1962; Sanahuja, Rio and Lede, 1965), that the plasma concentration of the limiting amino acid fell markedly within a few hours after rats ingested the test meal. Longenecker and Hause (1959) had found that the total plasma amino acid concentration in dogs was less after consuming a diet completely devoid of one amino acid than the total plasma amino acid concentration of controls fed casein protein. In contrast, the plasma amino acid concentration of animals on a protein-free diet were similar to those of animals consuming a basal diet (Harper, 1964, p. 110).

On the basis of these findings, and those of others, Harper hypothesized that imbalanced amino acid mixtures stimulate protein synthesis in the liver so that more of the limiting amino acid is thus retained in the liver of the imbalanced than the control group. If muscle continues to synthesize protein at a normal rate, using all amino acids, then the remaining circulating plasma amino acids could really become imbalanced. This apparent lack of homeostasis, i.e., the increasingly abnormal ratio of plasma amino acids to one another, could affect the appetite centers in the brain (Harper and Rogers, 1965).

Additionally, Leung (1964) found that 3 to 8 hours after feeding rats amino acid imbalanced diets the livers contained more free amino acids than those receiving a balanced diet. When liver tissue was exposed to increased

amounts of one EAA, increased incorporation of C^{14}-amino acids in a cell free system was observed (Hanking and Roberts, 1965).

Sanahuja and Rio (1967) also found an increased concentration of the dietary deficient amino acid in the liver and a corresponding decrease in the plasma when they pair-fed protein-depleted rats as follows: (1) a basal diet (10 per cent protein) (2) an imbalanced diet (basal diet + 3.1 per cent amino acid mix without threonine) and (3) a corrected diet (basal diet + 3.1 per cent amino acid mix with threonine). The livers of the rats consuming diet 2 contained more amino acid nitrogen, threonine and protein than those on diet 1. The concentration of threonine in the plasma was markedly lower on the imbalanced diet. The diets of groups 1 and 2 contained the same amount of threonine but diet 2 contained 3.1 per cent more protein. This could be an important factor since these animals were protein depleted. The diet of group 3 contained more threonine than group 2 but liver amino acid nitrogen, threonine and protein concentrations were not significantly different. The plasma threonine concentration of group 3 was three times that of group 2. The authors suggest that amino acid imbalance stimulated liver protein synthesis. It could be that total protein intake stimulated protein synthesis. It is commonly known that liver protein increases with added dietary nitrogen.

Sanahuja's data (Sanahuja and Rio, 1967) underscore the fact that both protein quantity (as an absolute value and as percentage in the diet) and quality affect protein content of the liver. It also raises the question of the difference

in the rate of protein biosynthesis in the liver when the total protein content of the amino acid deficient and control diet is the same.

The Mechanism of Altered Liver Protein Synthesis

It now appears that, in order to reduce the variables and get at the crux of the problem, the various sub-cellular particles involved in protein biosynthesis and its control must be examined. Mariani, Spadoni and Tomassi (1963) concluded that protein depletion increased the activity of the amino acid activating enzymes in rats. Fillios and Shaw (1967) found that hepatic RNA polymerase activity was inversely proportional to dietary protein level.

Sidransky, Staehelin and Verney (1964) noted enhanced leucine-C^{14} incorporation into liver proteins by cell-free preparations from fasted threonine-devoid animals. On the other hand, Fleck, Shepherd and Munro (1965) tube-fed an amino acid mix (-tryptophan) to fasted rats and one hour later, removed the livers and prepared cell-free extracts. When the cell-free particles were incubated with C^{14}-amino acids, the controls incorporated more C^{14}-amino acids than the particles from tryptophan-deficient animals.

On the basis of sucrose density gradient profiles, both found that the difference was due to a change in the ribosomes. Sidransky, Staehelin and Verney (1964) found proportionately more ribosomal polysomes from the experimental animals while Fleck, Shepherd and Munro (1965) found that the control animals had more polysomes. Wunner, Bell and Munro (1966) continued to investigate the mechanism of the effect of tryptophan deficiency and found that both polysome and oligosome preparations from control rats incorporated more

C^{14}-amino acids, per mg of protein, than the tryptophan-deficient. They also reported an increase in the post-microsomal fraction from the tryptophan-deficient. Webb, Blobel and Potter (1966) also reported that a single feeding of protein to fasted rats caused a shift in polysomes from lighter to heavier aggregates.

Recently, Sidransky et al. (1967) found that tryptophan alone was as effective in shifting the hepatic polyribosomal pattern from light to heavier as the complete amino acid mixture. Other amino acids--threonine, isoleucine and methionine--given singly, were not as effective as tryptophan. Thus it appears that trypotophan may play a special role in regulating the size of the polysomes and protein biosynthesis. This raises the question of the effect of other amino acid deficiencies on liver protein synthesis and the mechanisms controlling it.

Little is known about the effects of diets containing limited amounts of lysine, a key constituent of ribosomal nucleoproteins and of the histones which are associated with nuclear DNA. It has been proposed that these histones may play a role in controlling RNA synthesis (Busch and Starbuck, 1964). More importantly, there is only little information on the effect of inadequate consumption of a diet deficient in one or more EAA on the mechanism of protein biosynthesis in the liver.

Thus it is the purpose of this dissertation to examine the mechanism of altered protein synthesis caused by amino acid deficient diets using a lysine deficiency as a model.

MATERIALS AND METHODS

Animals

Eight groups of 22 day old female weanling Sprague-Dawley rats, weighing 34 to 62 grams (average, 49 grams) were used in the experiments. Twelve rats from two litters of six comprised a group. Littermates were paired according to weight on a 2 to 1 basis, i.e., 2 test animals to 1 control. The average weight of the 8 test animals was approximately the same as the average weight of the 4 control animals (Appendix 5 and Table 3).

The rats were housed individually in suspended galvanized wire mesh cages with test and control animals alternated to eliminate possible environmental variations. The animal room was maintained at approximately 78° F. Food and water were supplied ad libitum.

Diet

The two diets, test and control, were equal in all respects except protein quality (Table 1). Wheat gluten, a low quality protein comprised 20 per cent of the test diet which was replaced by casein, a high quality protein, in the control diet. The amino acid pattern of the two proteins is compared with the recommended amino acid pattern for rats in Appendix 1. It should be noted that the first limiting amino acid in wheat gluten is lysine; threonine content is

Table 1

Diet composition[a]

Component	Control diet g/100g	Test diet g/100g
Protein, casein	20	---
Protein, wheat gluten	---	20
Cornstarch	60	60
Cellulose (Alphacel)	6	6
Fat (corn oil)	10	10
Salt mixture U.S.P. XIV	4	4
Plus complete vitamin diet fortification mixture, equal amounts in each diet (Appendix 2).		
Approximate calories/100g	410	410

a. These diets were prepared by Nutritional Biochemicals Corporation; Cleveland, Ohio.

borderline. The contents of the vitamin mixture are itemized in Appendix 2.

All animals consumed the experimental diets for 33 days unless otherwise stated. The terminal age of the eight groups of animals and the corresponding number of experiments per group are summarized in Table 3 of the text and Appendix 5. Food intake was computed for two groups of rats. Since liver glycogen causes the ribosomal pellet to be soft (Korner, 1961), the rats were fasted for 17 hours before being killed.

Preparation of Subcellular Components

Cell sap and microsomal fraction

These components were prepared, with slight changes, according to the method of Corless and Gray (1967), a modification of the procedure used by Matthaei and Nirenberg (1961).

The liver from exsanguinated rats, decapitated with a guillotine, was quickly excised and placed in pre-weighed beakers containing ice cold Matthaei and Nirenberg (MN) buffer (Appendix 3). During all subsequent operations, the temperature was maintained between 2° and 5°C. All buffers were adjusted to the desired p^H (7.6) at 25°C, while stirring with a magnetic stirrer, and then brought to volume.

After the weight of the liver was determined, approximately 4 grams were cut into pieces and placed in 50 ml plastic centrifuge tubes containing a volume of MN buffer equal to twice the weight of the liver. The liver was

homogenized, using a Tri-R stirrer (Model S-63) fitted with a one inch teflon pestle, for 25 seconds using a single up and down stroke. After placing the glass mortar containing the tissue in ice to cool, homogenization was continued for 15 seconds using four strokes.

The tubes were then spun twice at 2°C in an International refrigerated centrifuge (Model B-20, angle head No. 870) for 10 minutes at 15,000 x g. The first supernate was used for the second centrifugation. After the second spin, the post-mitochondrial supernate (PMS) was aspirated and 5 ml was placed in each 10 ml cellulose nitrate ultracentrifuge tube. The tubes were then filled to capacity with MN buffer and spun at 128,000 x g for 2 hours in a Spinco Model L ultracentrifuge (50 rotor) to sediment the microsomal fraction.

The post microsomal supernatant fluid (cell sap), containing the enzymes and soluble ribonucleic acids (s-RNA), was aspirated and saved for later use. A 0.05 ml aliquot of the cell sap was diluted 40 times for protein determination. The microsomal pellet was washed twice with MN buffer and then, using a 5/8 inch teflon pestle, gently resuspended in 1 ml of MN buffer per gram of liver, making sure the centrifuge tube was ice cold at all times. The resuspended microsomal fraction was transferred to cold 50 ml centrifuge tubes and spun at 15,000 x g for 10 minutes to sediment aggregated material. Two 0.1 ml aliquots were diluted 10 times with water for protein and RNA determination.

Ribonucleoprotein particles (RNP particles)

RNP particles were prepared from the microsomal fraction using a modification of the Kirsch, Siekevitz and Palade (1960) method. The microsomal pellet, resuspended in 1 ml of Kirsch medium A buffer (K medium A, Appendix 3) per gram of liver was diluted five times with Kirsch medium B buffer (K medium B, Appendix 3). To the diluted suspension, 0.33 ml of 0.1 M $MgCl_2$ and 0.47 of a freshly made 3 per cent deoxycholate (DOC) solution, p^H 8.2 were added per gram of liver. The final concentrations of sucrose, $MgCl_2$ and DOC were 0.68M, 0.009M and 0.25 grams per 100 ml, respectively.

After 20 minutes, the mixture was transferred to chilled 50 ml centrifuge tubes and spun at 15,000 x g for 15 minutes to sediment aggregated material. The supernatant was then transferred to 10 ml ultracentrifuge tubes and spun in the 50 rotor at 128,000 x g for 60 minutes.

The supernatant fluid was immediately decanted, draining the tubes as thoroughly as possible. The ribosomal pellets were then rinsed twice with 1 ml each of K medium A plus mercaptoethanol, 0.006M (K medium A-SH), and gently resuspended in 0.2 ml K medium A-SH per gram of liver. After 0.05 ml aliquots were taken and diluted 40 times with water for protein and RNA determinations, the remainder was divided into 1 ml portions and frozen immediately at -15°C for subsequent use.

p^H 5 fraction

Cell sap was diluted with an equal volume of K medium B and, while stirring constantly with a magnetic stirrer, the p^H was gradually adjusted to 5.2 with 0.1 N acetic acid. After continuing to stir for 5 minutes, the mixture was allowed to stand for 10 minutes before sedimenting the precipitated enzymes by centrifuging at 8,000 x g for 10 minutes.

The pellet was rinsed with K medium B and resuspended in 0.2 ml MN buffer per gram of liver. This fraction contained all the enzymes necessary for protein synthesis in-vitro and s-RNA.

Purified enzymes

In an effort to gain more information on the possible difference in the mechanism of liver protein biosynthesis in these two dietary groups, beginning with Group IV, Experiment 6, the enzymes and s-RNA were separated and purified.

Takanami (1961) found that, after precipitating the p^H 5 enzymes from the cell sap, the remaining supernate showed a higher transferring activity (enzymes that transfer the amino acids from aminoacyl-s-RNA to ribosomes) than the p^H 5 fraction. After readjusting the p^H of the supernate to 7.6, he isolated these transfer enzymes by means of a diethylaminoethyl-cellulose (DEAE-cellulose) column. These enzymes adsorb to the column and can be eluted with 0.15 to 0.30 M KCl buffer. Subsequently, Takanami (1964) found

that all of the enzymes necessary for protein biosynthesis in-vitro could be isolated by applying the cell sap directly to the DEAE-cellulose column.

In order to conform more closely with the procedure used by Takanami, the method of preparing cell sap was changed slightly. Takanami used a homogenizing medium that was essentially the same as Kirsch's medium A with the exception of the sucrose concentration, 0.25 M versus 0.35 M, respectively. Since microsomes had been sedimented from 0.25 M sucrose in all previous experiments, it was desirable to continue using this molarity. Kirsch's medium A was therefore modified and the livers were homogenized in 2.4 ml per gram of liver. Ultracentrifuge tubes were filled to capacity with PMS which was prepared by centrifuging the liver homogenate twice at 15,000 x g for 10 minutes. Microsomes were sedimented and RNP particles were prepared as previously explained.

The top half of the post microsomal supernate was applied to a previously equilibrated DEAE-cellulose column, p^H 7.6. The column was then washed with 25 ml of the equilibrating buffer (0.03 M KCl; 0.02 M Tris, p^H 7.6) and the enzymes were eluted from the column with 25 ml of eluting buffer (0.3 M KCl; 0.02 M Tris, p^H 7.6). The average flow rate was 20 drops per minute. As the 0.3 M KCl buffer passes through, a distinct yellow band forms and most of the concentrated protein comes off in the 8th, 9th and 10th ml, depending on the height of the column and the flow rate. The most concentrated portions, usually 12 to 15 mg protein per ml, were combined and used in the incorporation mixtures. The protein concentration can quickly be estimated by

diluting an aliquot and determining the optical density (O.D.) at 280 mu in a spectrophotometer. Although the enzymes were eluted at room temperature, the column and all solutions were ice cold.

S-RNA preparation

S-RNA was isolated from the lower half of the post microsomal supernate by a modification of the phenol procedure employed by Ehrenstein and Lipmann (1961). Equal volumes of cell sap and freshly redistilled water-saturated phenol were combined and shaken for one hour in the cold. The mixture was centrifuged in Nalgene polyethylene tubes at 10,000 x g for 10 minutes, the aqueous phase aspirated and the phenol layer was washed with an equal volume of 0.001 M Tris buffer, p^H 7.6. To the combined water layers, one-tenth the volume of 20 per cent K Ac solution was added, and 2 volumes of 95 per cent ethanol at -20°C. This mixture was refrigerated at -15°C over night (Zubay, 1962) and then centrifuged in clear polycarbonate centrifuge tubes at -20°C at 15,000 x g for 15 minutes. All traces of ethanol were removed before resuspending the s-RNA in 0.1 ml of 0.1 M Tris per gram equivalent of liver. Aliquots for RNA determination were diluted 125 times with water and stored at -15°C for subsequent use.

DEAE-cellulose columns

DEAE-cellulose was washed according to the method outlined by Peterson and Sober (1964). One gram of dry DEAE-cellulose was allowed to sink into approximately 50 ml of 1 N NaOH which was decanted and replaced

with more NaOH solution until no more color remained. After the last NaOH was decanted, sufficient 1N HCl was added to make it strongly acid. This mixture was filtered immediately on No. 400 nylon mesh in a 4 inch Buchner funnel which was attached to a vacuum trap.

The DEAE-cellulose was washed free of acid with distilled water, again suspended in 1N NaOH, and finally washed free of alkali. After the last wash, it was suspended in buffer (0.03M KCl, 0.02 M Tris, p^H 7.6) and, while stirring constantly with a magnetic stirrer, the p^H was adjusted to 7.6 with 0.1 M KCl. During the next 24 hours, the buffer was changed several times to insure proper equilibration.

The DEAE-cellulose slurry was poured onto the column, allowed to settle, and approximately 40 ml of 0.03 M KCl buffer was passed through. The final height of the column was 10 cm and the flow rate, before applying the crude enzyme, averaged 40 drops per minutes.

Labelled Amino Acid Incorporation

The reaction mixture was similar to the Corless and Gray (1961) modification of that used by Matthaei and Nirenberg (1961). It contained the following in micromoles per milliliter: 100 Tris-HCl, p^H 7.6; 6.0 mercaptoethanol; 10 $MgAc_2$; 5.0 phosphoenolpyruvate (PEP); 0.03 each of cytosine triphosphate (CTP) and uridine triphosphate (UTP); 3.0 adenosine triphosphate (ATP); 0.5 μc C^{14}-amino acid or 2.0 μc phenylalanine-H^3; 25 micrograms pyruvate kinase. The following were also included in the reaction mixture in varying

concentrations, depending on the experiment: sucrose; KCl; guanosine triphosphate (GTP); non-radioactive amino acid carrier, to make a total of 0.03 micromoles L-amino acid, or 0.03 micromoles each of 20 L-amino acids; enzymes and s-RNA as cell sap, precipitated p^H 5 fraction, or separated on a DEAE-cellulose column and phenol extracted respectively; and ribosomes as microsomes or RNP particles. The exact concentration of each of the above components will be indicated with the results of the individual experiments.

Concentrated solutions of GTP, CTP, UTP, ion mix ($MgAc_2$, Tris and KCl) and amino acid mixes were prepared beforehand and stored at -15°C. Solutions of mercaptoethanol, PEP and ATP were prepared at the time of the reaction.

All chemicals included in the reaction mixture were combined and equal aliquots were pipetted into 10 ml polyethylene tubes, maintained at 2°C in an ice slurry. Enzymes and ribosomes, previously diluted to the required concentration, were then added to each tube. The 1 ml reaction mixtures were covered with parafilm and incubated for 30 minutes at 37°C in a Dubnoff shaker set at one stroke per second. All reactions were done in duplicate. After incubation, the reaction was stopped by immediately freezing in a CO_2-ethanol bath and then stored at -15°C until used for protein separation.

Preparation of Radioactive Proteins

The incorporation mixtures were washed and prepared for counting by the method of Siekevitz (1952). One milliliter of 10 per cent trichloracetic acid TCA) was added to the frozen sample, mixed thoroughly and allowed to

stand in ice for 30 minutes before sedimenting the precipitate at 1,000 x g for 10 minutes in a refrigerated centrifuge at 4°C. The samples were washed twice with 3 ml of cold 4 per cent TCA, extracted once with 2 ml of 4 per cent TCA for 15 minutes at 90°C, once with 3 ml of warm 95 per cent ethanol for 10 minutes, once with 3 ml of ethanol-ether-chloroform mixture (2:2:1) for 10 minutes and washed once with 2 ml of ether. After each treatment, the precipitate was sedimented by centrifugation at 4°C for 10 minutes. The speed was increased each time because the protein pellet becomes progressively softer with each purification.

The final protein sample was immediately air-dried while simultaneously dispersing the protein by mixing on a Vortex mixer. Two ml of 1 N NaOH was added to each sample and the mixture was heated for 10 minutes at 60°C to insure complete solution.

Aliquots, ranging from 0.2 ml to 1.0 ml of the protein solution were counted in 15 ml of a scintillation mixture (Bray, 1960) of toluene, dioxane and ethylene glycol monoethyl ether (Cellosolve), 1:3:3 (v/v); containing: 1 per cent 2,5-diphenyloxazole (PPO), 0.05 per cent 1,4-bis-2 (5-phenyloxazolyl)-benzene (POPOP), and 8 per cent naphthalene. Samples from Groups I through Group IV, Experiment 6, were counted in a Nuclear Chicago liquid scintillation counter (Model 8401). The settings for C^{14}-amino acids were as follows: gate high voltage, 1175 volts; data high voltage 900 volts. The window settings were Base-L_1, level 9.9. The remaining samples were counted in a Packard Tri-Carb scintillation spectrometer (Model 3310) with the following settings for

C^{14}-amino acids: window, 40-1000; gain, 7.75 per cent. The settings for phenylalanine-H^3 were: window, 40-200; gain, 100 per cent. In all cases, the standard error in counting was less than 2 per cent.

Corrections were applied for the background counts and protein loss during the Siekevitz wash. Since similar quenching was observed in all samples, no additional quench correction was applied to these data. The results are reported as counts per minute per mg of protein or counts per minute per mg of ribosomal RNA.

Protein Determination

Protein concentration in the various solutions was determined by the method of Lowry et al. (1951), employing the Folin phenol reagent. Diluted samples containing 25 to 40 gamma of protein per 0.2 ml were used; the concentration in the final volume (2.4 ml) was approximately 10.4 to 16.7 gamma per ml. The optical density (at 750 mμ) of the reaction mixture was read in a Beckman spectrophotometer (Model DB) using 1 cm pathlength cuvettes.

Since the color is not strictly proportional to the concentration (Lowry et al. 1951), calculations were made from a standard curve. Standard solutions were prepared with Labtrol, a serum protein (Appendix 4). It is particularly important to treat the standard solutions exactly the same as the samples.

RNA Determination

RNA concentration in the RNP particles was determined by the method of Scott, Fraccastoro and Taft (1956) as modified by Sox and Hoagland (1966), using an extinction coefficient of 32.3 O.D. per mg per ml. Four ml of cold 0.4 N $HClO_4$ were added to each 0.05 ml aliquot of resuspended ribosomal pellets which had been diluted 40 times with water (see preparation of RNP particles). After standing in ice for 10 minutes, the precipitate was sedimented by centrifuging for 10 minutes at 15,000 x g at 4°C. The pellets were then resuspended in 2.5 ml of 1 N KOH and held at 37°C for 30 minutes. After cooling in ice, 0.5 ml of 6 N HCl was added and the precipitated protein was removed by centrifugation for 10 minutes at 15,000 x g. One ml of the supernate was diluted 10 times and analyzed in a Beckman DB spectrophotometer.

RNA concentration in the s-RNA fraction was determined by the method of Ehrenstein and Lipmann (1961) using an extinction coefficient of 24 O.D. per mg per ml.

Separation of RNP Particles

The RNP particles were separated by means of sedimentation in the ultracentrifuge using a method similar to that described by Britten and Roberts (1960). One-half ml of K medium A without mercapto ethanol, containing 0.4 mg ribosomal RNA, was layered over 26 ml of a linear sucrose gradient (10 to 35 per cent, w/v) with the same ionic strength as the suspending medium. The gradients were spun for 150 minutes at 25,000 RPM at 4°C in a precooled

SW 25 swinging bucket rotor in a Spinco ultracentrifuge (Model L). The gradient was displaced upward through a 5 mm flow cell. Its absorbance at 260 mμ was assayed in a Beckman DU spectrophotometer. A Guilford recording attachment set at 0.25 amplitude was used to record the gradient profile.

Electrophoretic Separation of Purified Enzymes

Protein synthesizing enzymes from the livers of rats in Groups VII and VIII, were prepared by passing the cell sap through DEAE-cellulose (see Purified Enzymes). Aliquots of the concentrated enzyme fractions used in the incubation mixtures (the 9th and 10th ml combined) were separated electrophoretically according to a modification of the method of Davis (1964). Enzyme protein, 0.05 mg, was applied to the column (2 cm of stacking gel and 7 cm of separating gel). The protein was stained with Coomassie Brilliant Blue dye number R 250. The electropherograms were quantitated by densitometry using a Joyce Loebl Chromoscan (Model MK II) with a red filter.

RESULTS

Animal Response to Diets

Test animals consumed less food per day and gained less weight than the controls (Table 2). In recording the food consumption of two sample populations of rats, it was found that control animals ate an average of 14 grams of food daily while test animals averaged 10 grams. The rate of growth of the two dietary groups (wheat gluten versus casein) is illustrated in Figure 1. The mean body weight gain for six batches of rats kept on the diets for 33 days was 38 and 155 grams for the test and control animals respectively (Table 3). Test animals gained 24 per cent as much weight as the controls. The mean per cent weight gain was 67 for tests and 325 for controls. Thus, although the weight gain was much less, the animals on the deficient diet did continue to grow.

Control animals also utilized the food which they consumed more efficiently than the test animals. Average weekly food efficiency ratios (FER) are plotted in Figure 2. As the controls increased in size, the weekly FER values dropped. FER values remained relatively constant for those animals consuming the wheat gluten diet. During two separate experiments, the average FER of the two dietary groups were 0.36 for the controls and 0.15

Table 2

Average food consumed and weight gained by rats on wheat gluten (test) and casein (control) diets (for 28 days)

	Experiment[a]		Experiment[b]		Average	
	Control	Test	Control	Test	Control	Test
Wt. beginning (g)	53	53	48	48	50	50
Wt. end (g)	182	101	188	84	185	92
Wt. gain (g)	129	47	140	36	135	42
Food consumed (g)	401	289	380	263	390	276
F.E.R.	0.32	0.16	0.40	0.14	0.36	0.15
Food consumed/day (g)	14	10	14	9	14	10

a. These values are averages for 6 test and 6 control animals.

b. Eight test and 4 control rats comprised a group.

These measurements were made prior to the biochemical determinations, using other groups of rats.

Table 3

Summary of growth of six groups of rats

Group	III	IV	V	VI	VII	VIII	Average
Experiment	5	6&7	8	9	10&11	12	
			Control[a]				
Wt. - 22 days old (g)	48	48	54	48	46	46	48
Wt. - 55 days old (g)	228	187	205	201	200	201	204
Body wt. gain (g)	180	139	151	154	153	152	155
% body wt. gain	380	296	283	324	332	334	325
Liver wt. (g)	6.0	5.6	5.8	5.4	5.8	5.3	5.6
Liver/100g body wt. (g)	2.6	3.0	2.8	2.7	2.9	2.7	2.8
			Test[b]				
Wt. - 22 days (g)	49	48	56	48	46	46	49
Wt. - 55 days (g)	94	75	85	81	80	96	85
Body wt. gain (g)	46	30	28	38	34	50	38
% body wt. gain	95	58	52	66	76	108	67
Liver wt. (g)	2.4	2.3	2.4	2.2	2.5	2.8	2.4
Liver/100g body wt. (g)	2.6	3.0	2.9	2.7	3.1	2.9	2.9
			Ratio $\frac{\text{Test}}{\text{Control}}$				
Liver/100g body wt.	1.0	1.0	1.0	1.0	1.1	1.1	1.0
Wt. gain: % gain ratio	0.25	0.20	0.18	0.20	0.23	0.32	0.23

a. Average of 4 rats per group.

b. Average of 8 rats per group.

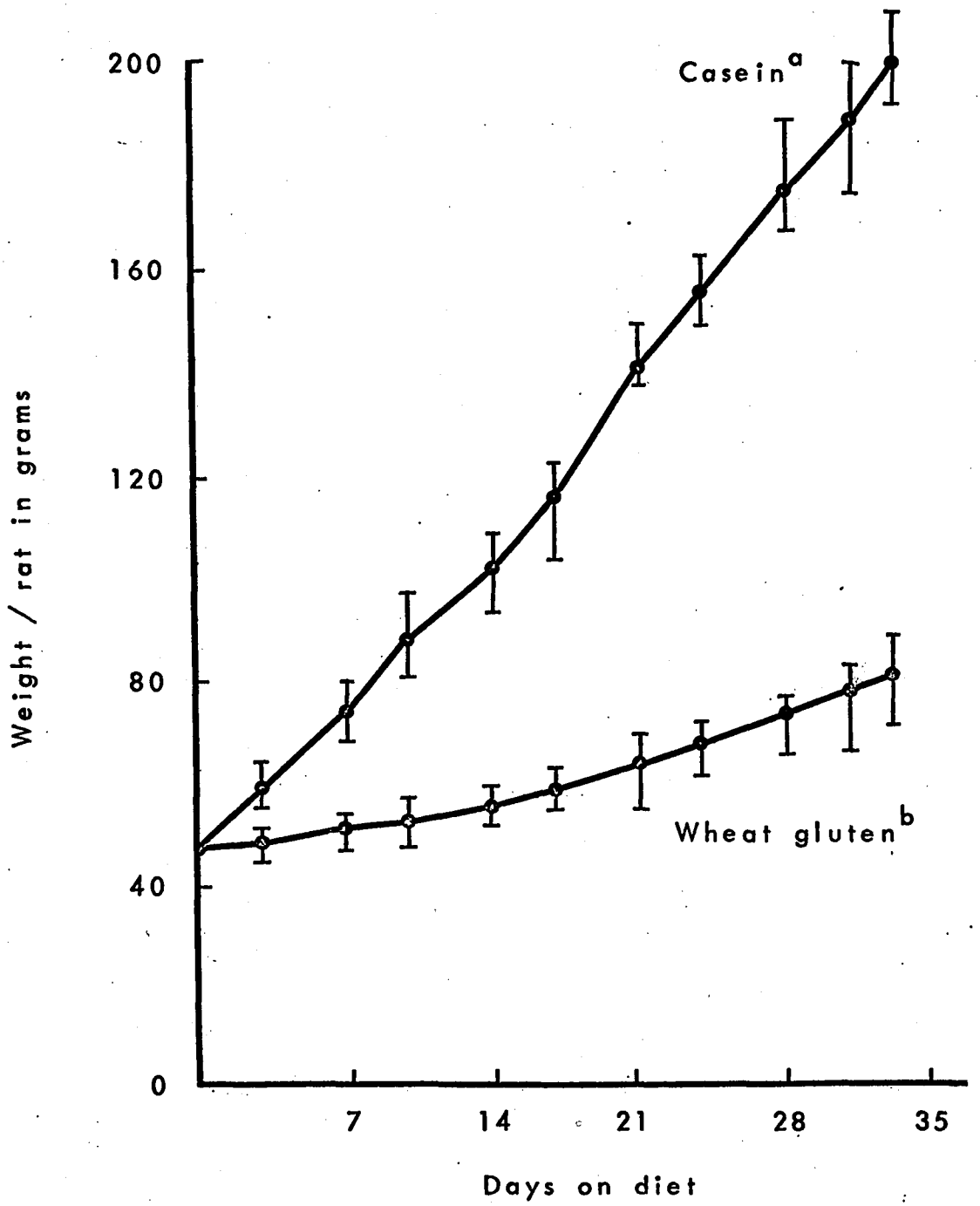

Figure 1. Growth curve for group VI

a) average for 4 rats b) average for 8 rats
I indicates range

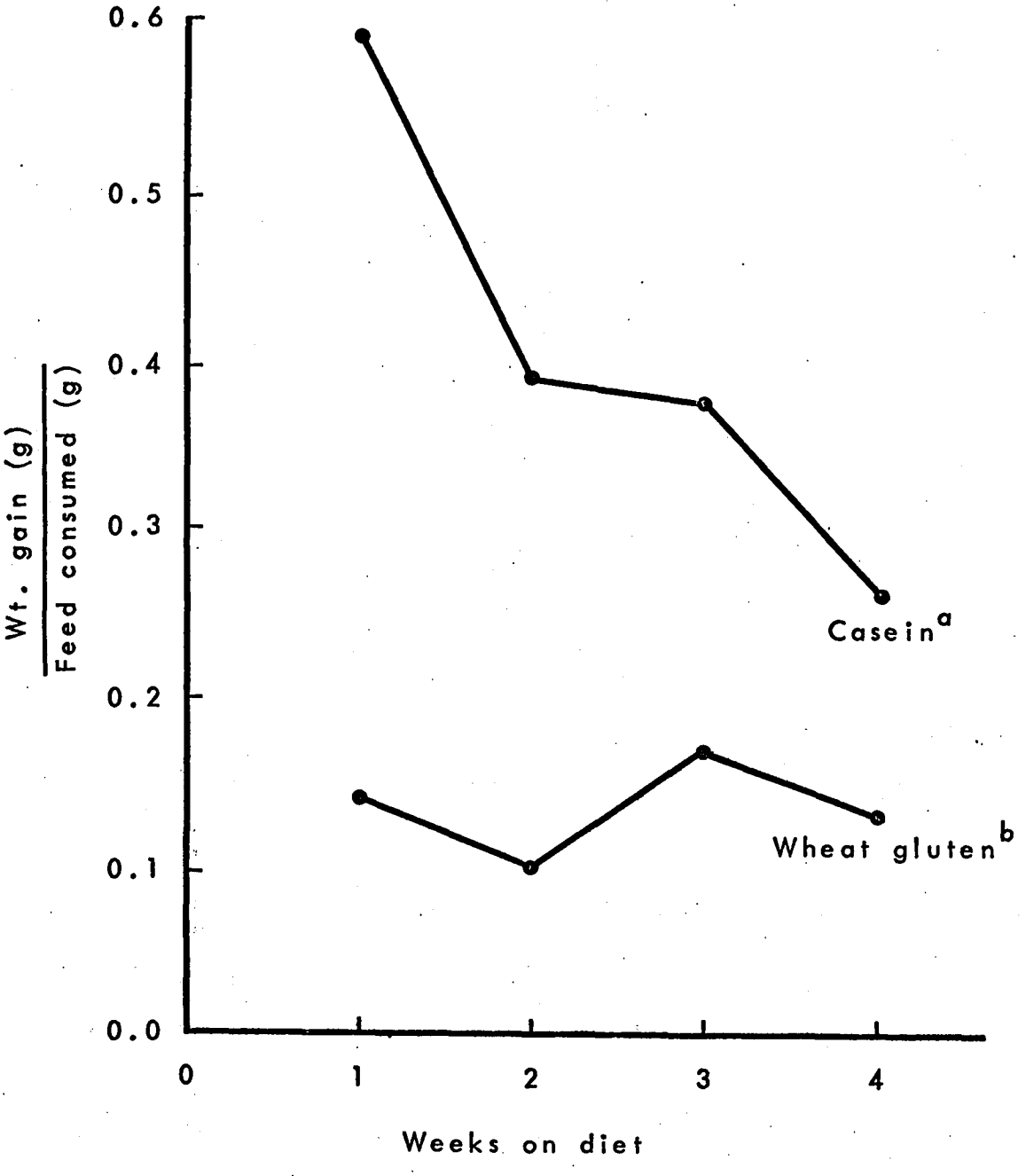

Figure 2. Weekly feed efficiency ratios

a) average for 4 rats b) average for 8 rats

for the tests. The test animals utilized the food which they consumed 42 per cent as efficiently as the controls.

When rats were kept on the diets for longer periods of time--the rats used in Experiments 1 through 4--the ratio of grams gained by tests versus controls increased with age (Appendix 5).

The gross appearance of the test animals was similar to that observed by Gray (1963). The coat was rough and test rats frequently lost hair from the hind legs. Culik and Rosenberg (1958) reported a similar response in lysine-deficient animals. The tail had reddish brown blotches and similar marks were often observed on the hind feet.

Internally, no free exudate was observed in the paritoneal cavity, but numerous fibrinous adhesions of the abdominal organs frequently involving the liver, were observed. The serosal surface of the intestine had a slightly yellowish cast and the contents of the intestine, particularly the large intestine, were extremely firm in consistancy and pale in color. No gross abnormalities were observed in the kidneys, spleen, adrenals or urinary tract.

Only one out of a total of 96 animals died during the entire study. The cause of death was not determined.

A Comparison of Liver Components

The livers of the animals on the wheat gluten diet were smaller than those of the animals on the casein diet. But, when grams of liver per 100 grams of body weight were compared, there was no difference in the liver size of the

two dietary groups. The average value for the tests was 2.9 grams of liver per 100 grams body weight and for the controls, 2.8 grams (Table 3).

The livers from test animals were friable and light colored with a yellowish cast. No measurements were made to determine the exact iron or ferritin content of the livers in question. However, from the general appearance of the test animal livers and the orange-red color of the ribosomal suspension (see below), it would appear that above normal amounts of iron may be present.

The characteristics of the microsomes used in Experiments 1, 2, and 3 are summarized in Table 4. The mean ratio of RNA/ protein was 0.16 and 0.13 for test and control microsomes respectively. These data suggest that there may be more RNA per unit of protein in the microsomes from test livers than there is in control microsomes. Sidransky, Staehelin and Verney (1964) reported that in rats force-fed threonine-devoid diets for three days total liver RNA increased 16 to 34 per cent.

With regard to the amount of ribosomal RNA and protein from the two dietary groups however, there appears to be no difference (Table 5). There is no significant difference in the ratio of ribosomal RNA to protein; the ratio for rats fed the wheat gluten was 0.77 and for those fed casein, 0.76.

Table 4

Microsomal characteristics

Experiment	Days on Diet	RNA / Protein		RNA / RNA + Protein	
		Control	Test	Control	Test
1	48	0.14	0.16	0.12	0.14
2	55	0.12	0.14	0.11	0.13
3	42	0.15	0.19	0.13	0.16
Average		0.13	0.16	0.12	0.14

Table 5

Characteristics of RNP particles from the two dietary groups

Group and Experiment No.	RNP-RNA/gram liver[a] mg		RNP-protein/gram liver[a] mg		RNA / Protein		RNA / RNA + Protein	
	C[b]	T[c]	C	T	C	T	C	T
IV - 6&7	1.03	1.09	1.32	1.38	0.78	0.79	0.44	0.44
V - 8	1.18	1.16	1.50	1.36	0.77	0.85	0.44	0.46
VI - 9	1.10	1.25	1.38	1.60	0.80	0.78	0.44	0.44
VII - 10&11	1.08	1.21	1.54	1.67	0.70	0.72	0.41	0.42
VIII - 12	1.19	1.17	1.60	1.66	0.74	0.70	0.43	0.41
Mean	1.12	1.18	1.47	1.53	0.76	0.77	0.43	0.43

a. These values are approximations since they were made by suspending the ribosomal pellets in 0.20 ml buffer per gram of liver rather than diluting to volume.

b. C = control animal on casein containing diet.

c. T = test animal on wheat gluten diet.

Liver In-vitro Protein Synthesis

Microsomal incorporation

Microsomes from test livers that were incubated with cell sap or p^H 5 enzymes incorporated significantly more leucine-C^{14} into protein than the same sub-cellular components from control livers (Table 6). When 1 mg cell sap protein was incubated with 1 mg microsomal protein, the test system incorporated 158 per cent more leucine-C^{14} than the control system in the same period of time.

An attempt was made to see if the increased activity could be attributed to the cell sap or the microsomes by crossing the fractions of test animals with those of the controls. Test microsomes incubated with control cell sap incorporated 104 per cent more leucine-C^{14} into TCA precipitated protein than the control system. There was no significant change when test cell sap was substituted for control cell sap. It should be noted, however, that control cell sap was 23 per cent less effective than test cell sap in the test system.

In order to eliminate some of the extraneous matter found in cell sap, the p^H 5 enzyme fraction was precipitated from the cell sap. When equal amounts of p^H 5 enzyme and microsomal protein were incubated together for 30 minutes, the test systems in Experiments 2 and 3 incorporated 77 per cent (in the former) and 54 per cent (in the latter) more leucine-C^{14} than the controls (Table 6). On the basis of these experiments, it appeared that--per mg

Table 6

Incorporation of L-leucine-C^{14} into microsomal preparations from the livers of rats fed a wheat gluten (T) or casein (C) diet

Experiment No.	Source of fraction		CPM/mg microsomal protein		Percentage of Control
			Duplicates	Average	
	Cell sap	Microsomes			
1	C	C	230/206	218	100
"	T	T	560/566	563	258
"	C	T	450/438	444	204
"	T	C	204/---	204	94
"	T[a]	T[a]	60/---	60	27
	p^H 5 Enz	Microsomes			
2	C	C	272/272	272	100
"	T	T	468/496	482	177
3	C	C	560/494	526	100
"	T	T	820/804	812	154
"	T[a]	T[a]	46/---	46	9

a. These samples are zero time incubations.

In addition to the chemicals listed in Materials and Methods, reaction mixtures contained the following in micromoles: 50 KCl, 25 sucrose, 0.002 L-leucine-C^{14}, 1 mg microsomal protein and 1 mg enzyme (cell sap or p^H 5) protein. Experiment 1 contained 0.06 micromoles GTP; Experiments 2 and 3, two-tenths. Weanling rats in Experiments 1, 2, and 3 consumed the diets for 48, 55, and 42 days respectively.

of microsomal protein--particles from the livers of the test animals incorporated more labelled leucine than particles from the control animals.

Ribosomal and p^H 5 enzyme incubations

It is possible that the cause of the increment observed in the previous experiments could have been associated with the microsomal membranes. Thus, ribonucleoprotein (RNP) particles were prepared from the microsomes. When these were incubated with the p^H 5 enzyme fraction, the previously observed increment in the test system persisted (Figure 3 and Table 7). The specific activity of the test system was 30 per cent greater than that of the control system. When fractions from different sources were crossed, the same pattern was observed as in the microsomal experiments noted above. Test ribosomes in the control system increased the rate of synthesis (21 per cent). There was no change in the rate of synthesis when test enzymes were incubated in the control system. As before, the specific activity of control enzymes incubated with test RNP was less (6 per cent) than that observed with the complete test system.

Enzyme concentration and leucine-C^{14} incorporation. In previous experiments, equal amounts of test and control p^H 5 enzymes were used in the incubation mixtures. However, it is possible that the proportion of the enzymes within this aliquot varies. Wettstein, Staehelin and Noll (1963) used 4 mg of p^H enzyme protein with approximately 1.1 mg RNP-RNA and stated that enzyme was always present in excess. In Experiment 4, increased

Figure 3

Effect of enzyme concentration on the incorporation of leucine-C^{14} during in-vitro protein synthesis

Item	Explanation of a, b, c, d Ratio of Protein, mg p^H 5 Enzyme:		RNP-Particle	Leucine Added Micromoles
a	1.0	:	1.0	0.002
b	2.0	:	1.0	0.03
c	2.56	:	1.0	0.03
d	5.1	:	1.0	0.03

All reaction mixtures contained the chemicals listed in Materials and Methods. Components which varied in concentration in the individual experiments are listed below.

Experiment 3, Group II. Rats consumed experimental diets for 43 days. Each reaction contained the following in micromoles: 50 KC1, 25 sucrose, 0.20 GTP. One or 2 mg enzyme protein and 1 mg RNP-protein were present per reaction.

Experiment 4, Group II. Rats consumed experimental diets for 53 days. Each reaction mixture contained the following in micromoles: 50 KC1, 150 sucrose, 0.20 GTP. 2.1 mg p^H 5-enzyme and 0.82 or 0.41 mg RNP-RNA were present per reaction.

Experiment 5, Group III. Rats consumed experimental diets for 33 days. Each reaction mixture contained the following in micromoles: 50 KC1, 125 sucrose, 0.20 GTP. 2.0 mg p^H 5-enzyme and 0.39 mg RNP-RNA were present per reaction.

	Source of Fractions Enzyme	 RNP Particles
▒	Control	Control
□	Test	Test
▨	Test	Control
⁘	Control	Test

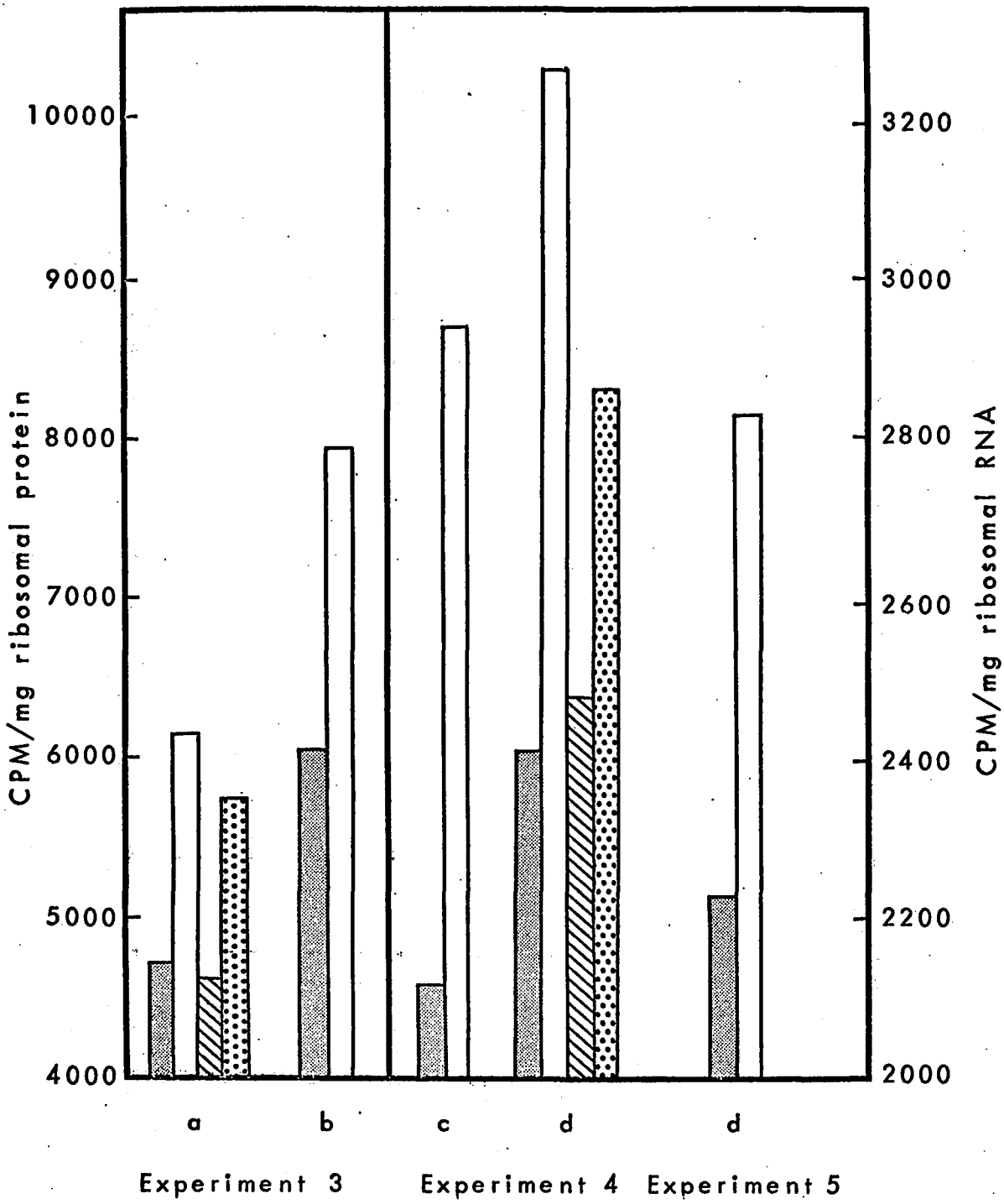

Figure 3. Effect of enzyme concentration on the incorporation of leucine-C^{14} during in-vitro protein synthesis.

Table 7

Summary of incubations using p^H 5 enzyme and RNP particles

Experiment 3					Experiment 4			
Particles								
p^H 5 Enzyme	RNP	Ratio[a]	CPM/mg Protein	Percentage of Control	Ratio[b]	CPM/mg RNA Duplicates	CPM/mg RNA Average	Percentage of Control
C	C	2:1	6054	100	2.56:1	2123/----	2123	100
T	T	2:1	7946	131	2.56:1	2900/2990	2945	139
C:	C	1:1	4762	100	5.1 :1	2410/----	2410	100
T	T	1:1	6178	130	5.1 :1	3284/3250	3267	136
C	T	1:1	5760	121	5.1 :1	2868/----	2868	119
T	C	1:1	4662	98	5.1 :1	2686/2483	2585	107
C[c]	C[c]				5.1 :1	Zero		

a. mg p^H 5 enzyme: mg RNP-protein.

b. mg p^H 5 enzyme: mg RNP-RNA.

c. Zero time incubation.

The conditions of these experiments are given in Figure 3.

amounts of enzyme were incubated with a fixed amount of RNP-RNA. The difference in the specific activity for the two dietary groups of rats persisted (Figure 3 and Table 8).

It should be noted that cold carrier leucine was added to the incubation mixtures in Experiment 4 but this did not affect the difference between the two systems.

Effect of adding 19 free amino acids to incubation mixtures. As noted in the Introduction, Leung (1964) found more free amino acids in the livers of rats eight hours after feeding amino acid imbalanced diets. It is possible that the difference in the incorporating capacity of the two systems could be due to a difference in endogenous free amino acid concentration. The effects that have been reported in the literature concerning the addition of amino acids to the incubation mixture are contradictory. In this experiment, the addition of 0.03 micromoles of each of the 19 free amino acids to each incubation increased the specific activity of the control system more than it did the specific activity of the test system (16 versus 5 per cent) (Figure 4 and Table 8). The difference between test and control systems, in their ability to incorporate leucine-C^{14} into ribosomal protein, was reduced with increased free amino acid concentrations. In Experiment 5, without additional amino acids, the specific activity of the test system was 27 per cent greater than that of the control system. With additional amino acids, the difference was reduced to 16 per cent (Table 8).

Table 8

A comparison of leucine-C^{14} incorporation into liver proteins with and without exogenous free amino acids in the incubation mixture[a]

Particles[b]		CPM/mg ribosomal RNA		Percentage
Enzyme	RNP	Duplicates	Average	of Control
		Without additional 19 free amino acids		
C	C	2244/2218	2232	100
T	T	2794/2878	2836	127
		With additional 19 free amino acids		
C	C	2568/2593	2580	100
T	T	2903/3079	2991	116
C	T	3096/3220	3158	122
T	C	2794/2520	2657	103

a. Experiment 5.

b. The ratio of mg enzyme protein to mg RNP-RNA was 5.1:1 in all reactions. Details of the incorporation mixtures are given with Figure 3.

Figure 4

Effect of 19 additional amino acids on the incorporation of leucine-C^{14} during in-vitro protein synthesis

Explanation of a, b, c, d

Ratio of particles (mg enzyme protein: mg RNA)	a	b	c	d
p^H 5 Enz: RNP-RNA	5.1:1	5.1:1		
Enz:s-RNA:RNP—RNA			1:0.2:1	1:0.2:1
L-amino acids added (u moles)	0.03 leucine	0.03 ea of 20	0.03 leucine	0.03 ea of 20

Experiment 5: Details of the incorporation are the same as those listed in Figure 3.

Experiment 6: Rats consumed experimental diets for 33 days. In addition to those chemicals listed in Materials and Methods, each reaction contained the following in micromoles: 65 KC1, 70 sucrose, 0.2 GTP, 0.5 mg enzyme protein, 0.1 mg s-RNA, and 0.5 mg RNP-RNA were present in each reaction.

Dotted lines indicate the highest and lowest individual values (CPM/mg RNA).

	Source of Fractions	
	Enzyme	RNP Particles
▒	Control	Control
□	Test	Test
▨	Test	Control
⁘	Control	Test

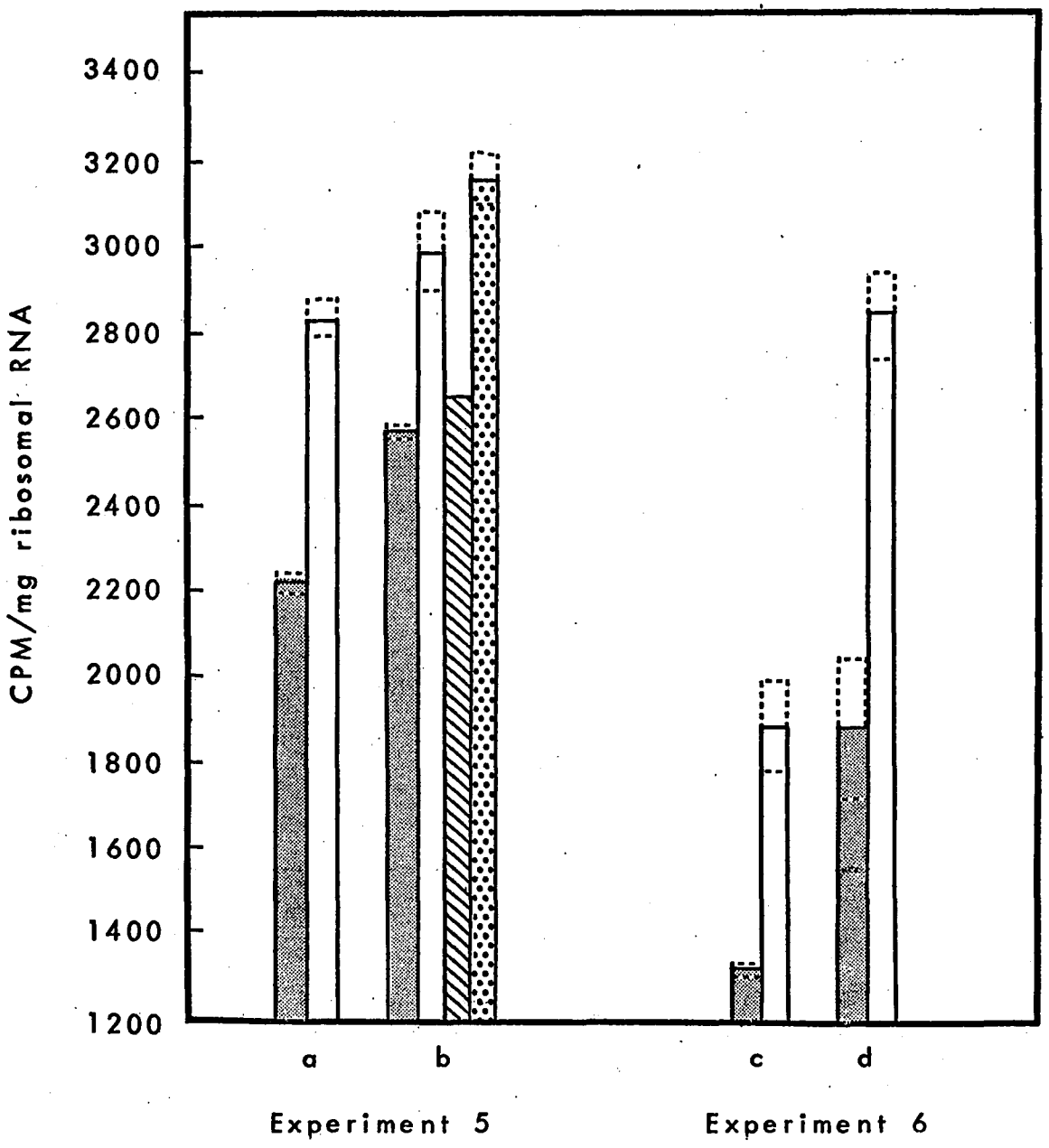

Figure 4. Effect of 19 additional amino acids on the incorporation of leucine-C^{14} during <u>in-vitro</u> protein synthesis.

When the particles from the two systems were crossed, the effect of test enzymes on the control system was no longer apparent. We can therefore conclude that with optimum amounts of enzyme and free amino acids there is still a difference in synthesizing capacity which may be attributed to the ribosomes of the two dietary groups.

Effect of separating enzymes and s-RNA

It is possible that the s-RNA which separates out with the precipitated p^H 5 enzymes is not present at an optimum concentration relative to the enzymes. Therefore, in order to be able to control the amount of s-RNA used in the reaction mixture and to purify further the enzymes, s-RNA was extracted from the cell sap by the phenol method described above and the enzymes were eluted from a DEAE-cellulose column with 0.3 M KCl buffer.

Whereas Takanami (1964) used an 8 to 1 ratio of ribosomes to s-RNA in his incorporation studies, a 5 to 1 ratio was employed in this study. Based on the experiments reported in Table 9, it appears that ours is an optimum ratio.

When enzyme protein, s-RNA and ribosomal RNA were added to an incubation mixture--without additional free amino acids--in a 1: 0.2: 1 ratio, the test systems incorporated 42 per cent more leucine-C^{14} than the control system (Table 9 and Figure 4). The addition of amino acids to the system increased this difference between test and control systems to 52 per cent. Synthesis within the control system increased 42 per cent when free amino

Table 9

Relationship of enzymes, s-RNA and free amino acid concentrations to protein synthesis[a]

Particles			CPM/mg ribosomal RNA		
Enzyme protein mg	s-RNA mg	Ribosomal RNA mg	Duplicates	Average	Percentage of Control
With 0.03 micromoles of each of 20 L-amino acids					
Control					
0.5	0.1	0.5	1890/----	1890	100
0.5	0.2	0.5	2108/2018	2064	109
1.0	0.1	0.5	2692/2760	2726	144
1.0	0.2	0.5	2530/2550	2540	134
Test					
0.5	0.1	0.5	2854/----	2854	152
1.0	0.2	0.5	3390/3304	3348	182
Without the 19 additional L-amino acids					
Control					
0.5	0.1	0.5	1322/1344	1332	100
Test					
0.5	0.1	0.5	1790/2000	1890	142

a. Experiment 6. See description accompanying Figure 4 for detailed conditions of the experiment.

acids were added and 51 per cent within the test system. This difference in percentage increment (42 versus 51) is within the experimental error of the method. Therefore, on the basis of these experiments, it appears that adding additional free amino acids to either system increased the total leucine-C^{14} incorporation 40 to 50 per cent.

Moreover, doubling the ratio of enzyme protein and s-RNA to ribosomal RNA increased the specific activity of the control system 34 per cent and the test system, 17 per cent.

With enzymes and ribosomes both at limiting concentrations (1 to 1) and s-RNA at optimum concentration (0.2 mg RNA per mg enzyme), crosses were made among the liver fractions from the two dietary groups of animals to see if the previously noted conditions still prevailed when more purified fractions and exogenous free amino acids were used in the incubation mixtures. It appears that both the enzyme and the RNP fractions contribute to the increased leucine-C^{14} incorporation of the test system (Table 10). In two separate experiments, the specific activity of the test system was 51 and 46 per cent greater than that of the control system. Substituting test s-RNA into the control system (C-T-C) had no effect while the percentage increase was the same (31 per cent) when test RNP (C-C-T) or test RNP and s-RNA (C-T-T) were substituted in the control system. When test enzymes (T-C-C) or test enzymes and s-RNA (T-T-C) were substituted into the control system, leucine-C^{14} incorporation also increased (136 and 132 per cent respectively).

Table 10

Localization of stimulatory factors in liver protein synthesis using three separate cell fractions

Source of particles used in synthesis Enz--s-RNA--RNP	CPM/mg Ribosomal RNA					
	Experiment 6[a]			Experiment 7[a]		
	Duplicates	Average	Percentage of Control	Duplicates	Average	Percentage of Control
C-C-C	1890/----	1890	100	4013/3660	3836	100
T-T-T	2854/----	2854	151	5670/5532	5601	146
T-C-C	2612/2528	2570	136	5900/5907	5904	154
C-T-C	1856/2094	1974	104			
T-T-C				4891/5203	5047	132
C-T-T	2486/2244	2364	125	4244/----	4244	131
C-C-T				4256/4046	4151	131
C-C-C[b]	98/--	98	5	165/ 229	194	5
-----T	193/---	193	10			

a. See description accompanying Figure 4 for detailed conditions of the experiments. The conditions were the same for experiments 6 and 7. All of the reactions contained 0.03 moles of each of 20 L-amino acids.

b. Zero time incubation.

Samples from Exp. 6 were counted in a Nuclear Chicago liquid scintillation counter; those from Exp. 7 were counted in a Packard Tri-Carb liquid scintillation spectrometer which had a higher percentage efficiency.

Increasing concentrations of enzyme and s-RNA (5 parts of enzyme to 1 part s-RNA) were incubated with a constant amount of RNP-RNA--0.32 mg RNA in Experiment 10 and 0.34 mg in Experiment 12 (Table 11). The pattern for the two systems was the same (Figure 5). Incorporation of leucine-C^{14} increased linearly until the enzyme protein concentration was the same as that of RNP-RNA. At this point the uptake of the amino acid apparently decreased. Maximum incorporation of leucine-C^{14}, per mg of RNP-RNA, was reached when the enzyme protein to RNA ratio was 4 to 1. When the enzyme to RNA ratio was greater than 4 to 1 in the incubation mixtures, the specific activity decreased slightly.

A Lineweaver-Burk analysis of the results showed that the V_{max} of the test system increased from that of the control system (Figure 6). However, the K_m remained the same. This of course, could mean that the observed alterations in activity might be the result of irreversible (non-competitive) changes in the reacting components. These observations were obtained in both Experiments 10 and 12.

Incorporation of other labelled amino acids

In an effort to determine whether the observed changes were specific for leucine or of a general nature, other radioactive amino acids--lysine, alanine and phenylalanine--were used in the incubation mixtures. The results of three separate experiments (numbers 8, 9 and 11) are summarized in Table 12 and Table 13. Data on the individual incubations are given in Appendix 6 and Appendix 7.

Table 11

Effect of increasing enzyme and s-RNA concentrations on leucine-C^{14} incorporation

Ratio of Particles	Experiment 10[a]			Experiment 12[b]			
	CPM/mg RNP-RNA		Percentage			Percentage	Average Percentage
Enzyme:s-RNA:RNP-RNA	Control[c]	Test[c]	of Control	Control[c]	Test[c]	of Control	of Control
6: 1.2: 1	----	----	----	6068	7715		
				6609	7715	122	122
5: 1.2: 1	6651	8250	117				117
	6919	7700		----	----	---	
4: 0.8: 1	6950	8325		6820	7903		
	7225	8662	120	6527	8114	120	120
2: 0.4: 1	6088	7250		5468	----		
	5612	7600	127	5562	6938	126	126
1: 0.2: 1	4075	5588		3563	5316		
	4062	5712	139	3810	5363	145	142
0.5: 0.1: 1	2612	3200					
	2688	3412	125	----	----	---	125
0.4: 0.08:1	----	----	---	2140	3011		
				2117	3011	142	142

Table 11 (Continued)

Effect of increasing enzyme and s-RNA concentrations on leucine-C^{14} incorporation

Ratio of Particles	Experiment 10[a]			Experiment 12[b]			
	CPM/mg RNP-RNA						
Enzyme:s-RNA:RNP-RNA	Control[c]	Test[c]	Percentage of Control	Control[c]	Test[c]	Percentage of Control	Average Percentage of Control
1: 0.2: 1[d]	37		1	29		1	1
1: 0.2: --	35		1				1
-: ---: 1	49		1				1

a. Concentration of particles per reaction: Enzymes, 0.16 to 1.6 mg/0.3 ml; s-RNA, 0.032 to 0.32 mg/0.3 ml; RNP-RNA, 0.32 mg/0.1 ml.

b. Concentration of particles per reaction: Enzymes, 0.136 mg to 2.03 mg/0.3 ml; s-RNA, 0.027 to 0.41 mg/0.3 ml; RNP-RNA, 0.34 mg/0.1 ml.

c. Duplicates.

d. Zero time incubation.

Each reaction contained the following in micromoles: 95 KCl, 35 Sucrose, 0.2 GTP, 0.03 of each of the 20 L-amino acids including 0.5 μc leucine-C^{14}.

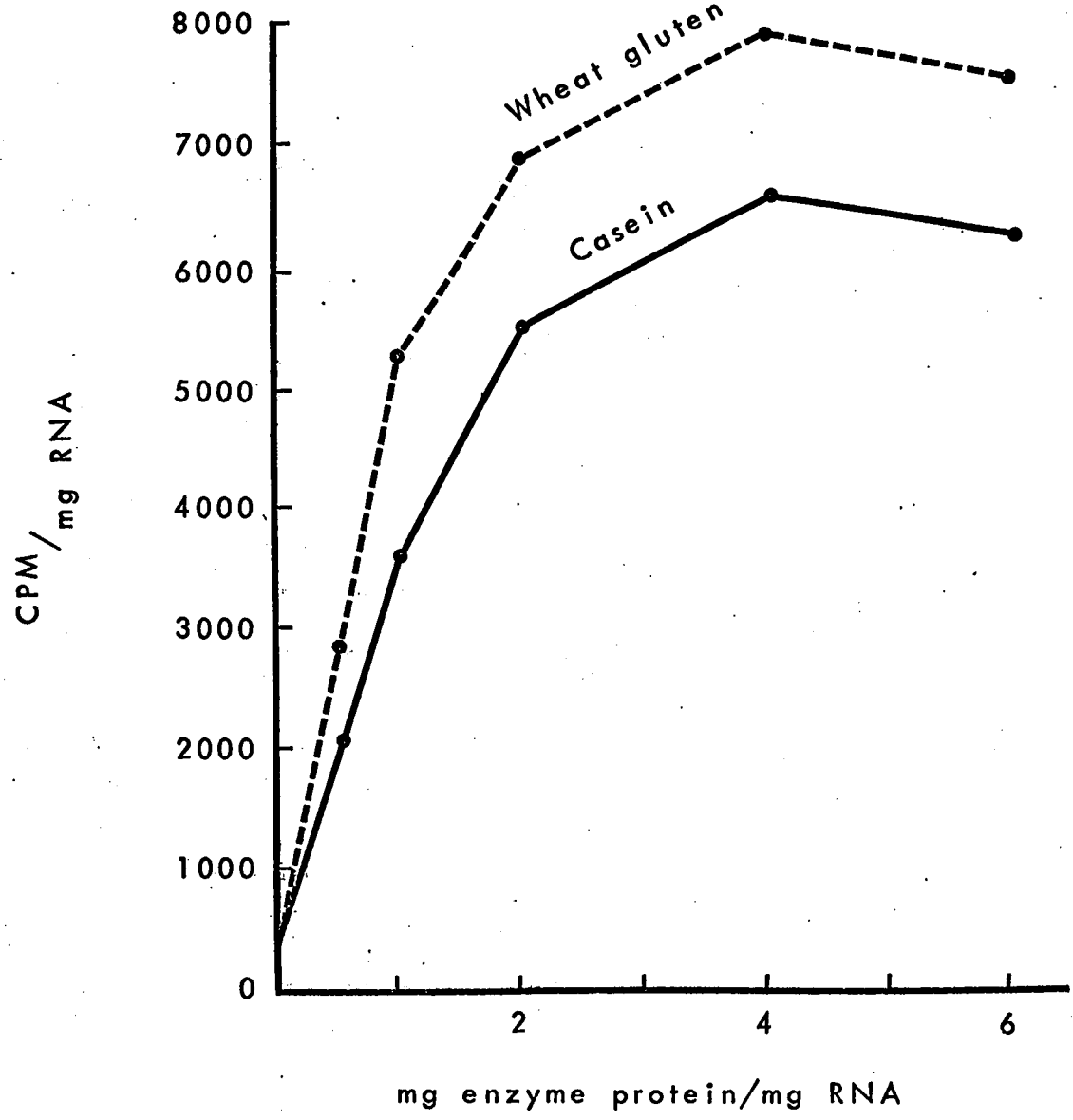

Figure 5. Effect of enzyme and s-RNA concentration on leucine-C^{14} incorporation by liver RNP fractions from test (Wheat gluten) and control (casein) fed rats, experiment 12.[a]

a) Details of incubation mixtures are given in table 11.

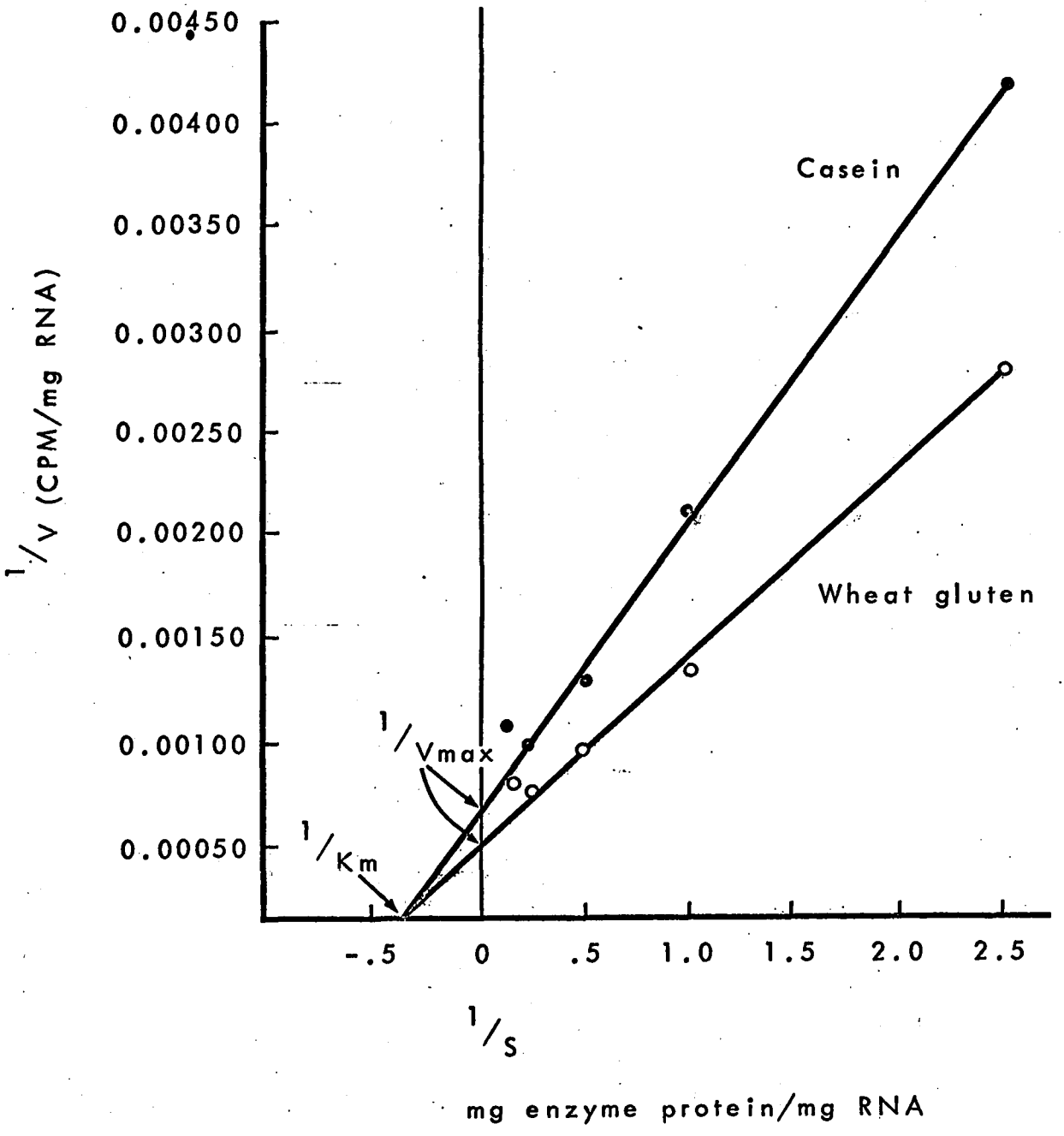

Figure 6. Lineweaver-Burk analysis of data from experiment 12.

Table 12

Ratios[a] of labelled amino acids incorporated into liver proteins of rats on the control diet[b]

Experiment No.	Amino acid	labelled with C^{14} Leucine	Lysine	Alanine	Phenylalanine	Phenylalanine-H^3
8	Leucine-C^{14}		0.65	0.95	----	0.55
9	"		0.74	0.99	0.56	----
11	"		0.73	0.89	0.53	----
Av.			0.71	0.94	0.54	0.55
8	Lysine-C^{14}	1.53		1.46	----	0.85
9	"	1.35		1.33	0.75	----
11	"	1.37		1.21	0.73	----
Av.		1.42		1.33	0.74	0.85
8	Alanine-C^{14}	1.05	0.68		----	0.58
9	"	1.01	0.75		0.56	----
10	"	1.13	0.82		0.60	----
Av.		1.06	0.75		0.58	0.58
8	Phenylalanine-C^{14}	----	----	----		
9	"	1.79	1.33	1.77		
10	"	1.88	1.37	1.67		
Av.		1.84	1.35	1.72		
8	Phenylalanine-H^3	1.81	1.18	1.72		

a. Ratios were calculated by dividing the specific activities for the amino acids listed horizontally by those listed vertically.

b. Individual incorporation values and incubation mixtures for the 3 experiments are presented in Appendix 6.

Table 13

Ratios[a] of labelled amino acids incorporated into liver proteins of rats on the test diet[b]

Experiment No.	Amino acid	Labelled with C^{14} Leucine	Lysine	Alanine	Phenylalanine	Phenylalanine-H^3
8	Leucine-C^{14}		0.61	0.98	----	0.55
9	"		0.84	1.23	0.67	----
11	"		0.74	0.94	0.51	----
Av.			0.73	1.05	0.59	0.55
8	Lysine-C^{14}	1.66		1.62	----	0.91
9	"	1.19		1.48	0.79	----
11	"	1.36		1.28	0.69	
Av.		1.40		1.46	0.74	0.91
8	Alanine-C^{14}	1.02	0.61		----	0.56
9	"	0.81	0.68		0.54	----
11	"	1.06	0.78		0.54	----
Av.		0.96	0.69		0.54	0.56
8	Phenylalanine-C^{14}	----	----	----		
9		1.50	1.26	1.85		
11		1.97	1.45	1.86		
Av.		1.74	1.36	1.86		
8	Phenylalanine-H^3	1.83	1.10	1.72		

a. Ratios were calculated by dividing the specific activities for the amino acids listed horizontally by those listed vertically.

b. Individual incorporation values and incubation mixtures for the three experiments are presented in Appendix 6.

It appears that there is no significant difference among the several comparisons of the two dietary groups. Regardless of the amino acid used in the incorporations, the specific activity of the test system was consistently higher than that of the control--25, 28, 39 and 32 per cent for leucine, lysine, alanine and phenylalanine respectively (Table 14).

Sucrose Gradient Patterns of Liver RNP

Ribosomes from the livers of experimental animals fed wheat gluten and those fed casein diet were separated in a sucrose gradient. Results from two experiments (Table 15) indicated that the ribosomal distribution patterns for the two groups of animals were similar. The ribosomal fractions (Figure 7) have been designated as follows: A, monomers; B, dimers; C and D, ergosomes (aggregates larger than dimers). This corresponds with the ribosomal aggregates of Sidransky, Staehelin and Verney (1964), using a 10 to 34 per cent sucrose gradient. In the control animal, 37 per cent of the ribosomes were ergosomes, 33 per cent monomers and 30 per cent dimers. In the test animal, 35 per cent were ergosomes with monomers (38 percent) and dimers (28 per cent) making up the balance. The sucrose gradient patterns of ribosomes from Group VII are shown in Figure 7. The possible meaning of these results will be elaborated upon in the discussion. However, it is apparent that more work needs to be done in this area in order to arrive at any firm conclusions.

Table 14

In-vitro liver protein synthesis by particles from rats fed wheat gluten (T) or casein (C) using 4 different amino acids[a]

Amino acid		Experiment 8 CPM/mg RNA	Experiment 8 Percentage of Control	Experiment 9 CPM/mg RNA	Experiment 9 Percentage of Control	Experiment 11 CPM/mg RNA	Experiment 11 Percentage of Control	Mean Percentage of Control
Leucine-C^{14}	T	5190		4255		5740		
	C	4086	127	3726	114	4240	135	125
Lysine-C^{14}	T	3124	117	3576	129	4236	137	128
	C	2665		2768		3100		
Alanine-C^{14}	T	5082		5248		5420		
	C	3896	130	3684	142	3764	144	139
Phenyalanine-C^{14}	T			2832		2916		
	C			2077	136	2260	129	132
Phenylalanine-H^3	T	2840	125					125
	C	2262						

a. Individual incubation mixtures and incorporation values for the 3 experiments are given in Appendix 6.

Table 15

Fractions[a]

A comparison of liver ribosomal sucrose density gradient[b] fractions

Group	A Percent[c]	B Percent[c]	C Percent[c]	D Percent[c]	C + D Percent[c]
			Control		
VII	30	32	12	26	38
VIII	37	28	11	24	35
Average	33	30	12	25	37
			Test		
VII	35	32	12	21	33
VIII	40	23	12	24	36
Average	38	28	12	23	35
			Ratio: $\frac{\text{Test}}{\text{Control}}$		
VII	1.17	1.00	0.98	0.82	0.87
VIII	1.09	0.84	1.03	1.04	1.04
Average	1.13	0.92	1.00	0.93	0.95

a. The fractions are designated in Figure 7.

b. Ten to 35 per cent sucrose gradient. The method is explained in the Materials and Methods section.

c. Percent = fraction of total square inches in pattern.

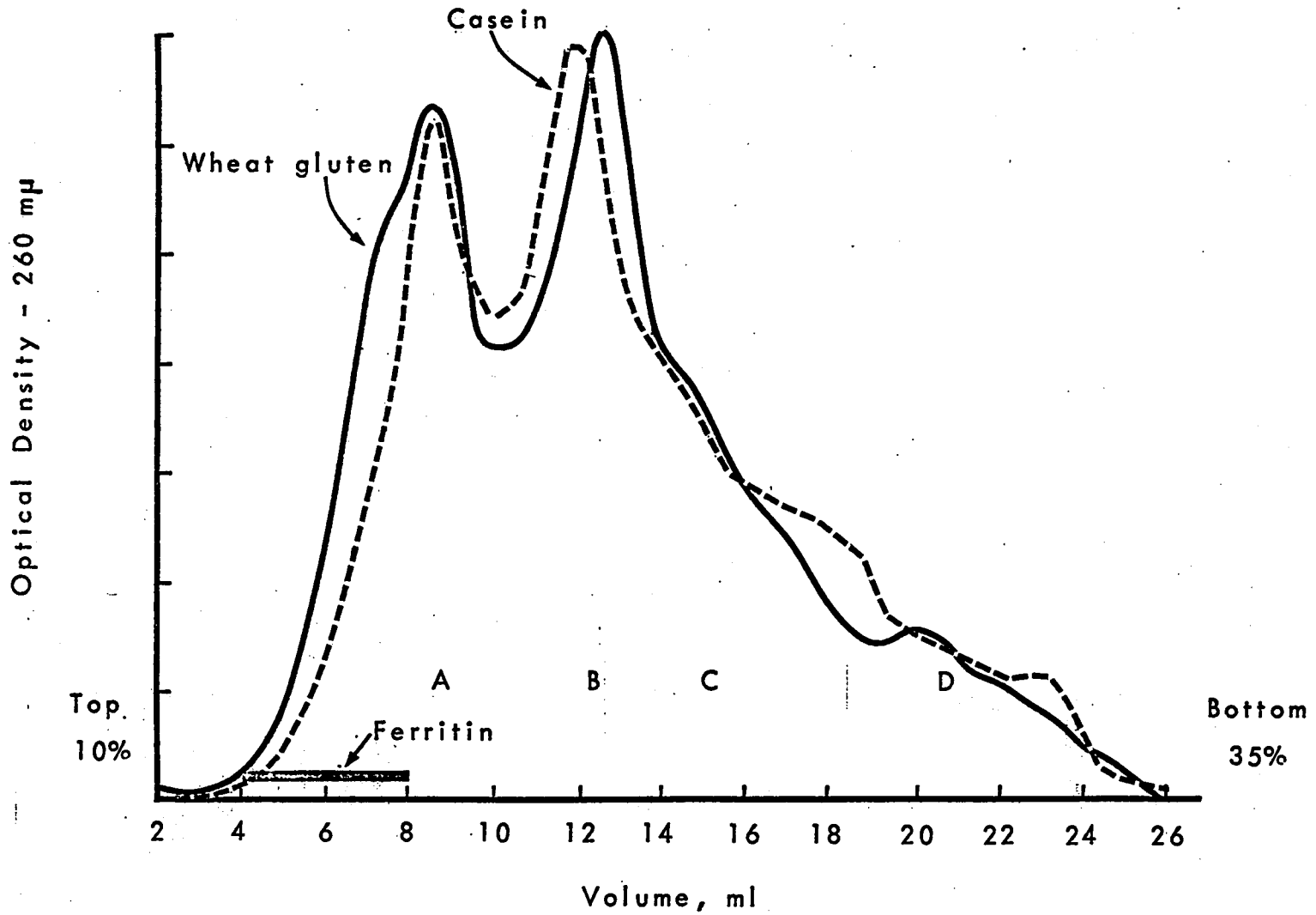

Figure 7, Sucrose gradient patterns of liver RNP-particles

DISCUSSION

General Considerations

In our experiments, the observed reduced food intake, growth rate and feed efficiency ratio (FER) in rats consuming wheat gluten protein are characteristic symptoms of amino acid imbalance (see Harper, 1964, p. 93). The test animals, consuming the wheat gluten protein gained steadily but at a slower rate than the controls. The FER did not decrease with age as it did in the controls. It appears that in the test animals maturity was delayed. In agreement with our lower FER values for the wheat gluten fed rats, Allison et al. (1964) reported that twice as much wheat gluten nitrogen as casein was needed to maintain body weight in weanling rats and three times as much wheat gluten was utilized to produce a 50 gram weight gain. It is also commonly known that lysine, a key constituent of ribosomal nucleoproteins (Cohn, 1965) and the histones, is required for growth (see Jansen, 1962).

In agreement with Sanahuja and Rio (1967), it was found that the size of the liver, grams per 100 grams body weight, of rats consuming an amino acid imbalanced diet was not significantly different from the controls that were fed the casein diet containing the same amount of protein. The gross appearance of the livers from test animals was similar to that frequently described for the livers of protein-calorie malnourished children (see Viteri et al., 1964,

p. 537 and Platt, Heard and Stewart, 1964). Thus is appears, that in our experiments, the gross findings were similar to those reported by others studying the effects of dietary amino acid imbalanced proteins in rats.

In-vitro C^{14}-Amino Acid Incorporation

The results of these experiments demonstrate that the protein fractions from the livers of rats fed an imbalanced amino acid diet incorporated labelled amino acids at a faster rate than control animals. This finding is in keeping with the results of in-vivo studies discussed at length in the Introduction (Stirpe and Schwarz, 1963, Nimni and Bavetta, 1961; Sidransky and Verney, 1964; Hartman and King, 1967; Harper et al., 1964; etc.).

Microsomal fraction and cell sap

The ratio of RNA to protein of the liver microsomal fractions from the two dietary groups of animals in our studies were in the same range as Palade and Siekevitz (1956) reported as average values for microsomes from normal rats (0.15 to 0.16). We found that the average ratio of RNA to protein was 0.13 for the controls versus 0.16 for the tests. These results suggest that the microsomal fraction from the amino acid deficient rats may have contained relatively more RNA. As mentioned earlier in the Results Section of this dissertation, Sidransky, Staehelin and Verney (1964) found that in rats force-fed threonine-devoid diets for three days total liver RNA increased 16 to 37 per cent. Also, Hoagland, Scornik and Pfefferkorn (1964) found that the

microsomal fraction from regenerating liver had more RNA per gram equivalent of liver than the normal rats.

It appears that the increased incorporation of leucine-C^{14} (158 per cent) by the protein synthesizing components isolated from the livers of rats consuming a wheat gluten diet may be due primarily to the microsomal fraction (Table 6). However, there is evidence that alterations in the cell sap may also contribute to the difference between the two systems. Test cell sap incubated with control microsomes had no effect on amino acid incorporation. But control cell sap incubated with test microsomes (C-T) incorporated 104 per cent more than the control system (C-C). If the increase in the rate of leucine-C^{14} incorporation by the test system was due entirely to a change in the microsomal fraction, the specific activity of control cell sap incubated with test microsomes (C-T) and the test system (T-T) should be the same. The fact is however, the incubations containing control cell sap and test microsomes incorporated 21 per cent less than the test system (T-T). This lower activity, per mg protein, could be due to a number of factors.

It is possible that the test cell sap contains more free amino acids. Leung (1964) found that the livers of animals fed an amino acid imbalanced diet contained more free amino acids eight hours after feeding than rats that consumed a good quality protein. If this were the case, the control cell sap might not contain sufficient amino acids for the test microsomes to act with the same efficiency as with the test cell sap. This could account for the lower activity of the C-T as compared to the T-T system. Similarly, the higher

concentration of amino acids in the test cell sap could be greater than can be used by the control microsomes which may be functioning optimally with its control cell sap concentration. Therefore we do not see any stimulation of the T-C over the C-C system.

Substances that stimulate and inhibit protein biosynthesis have been found present in liver cell sap (Munro, Jackson and Korner, 1964; Bont, Razelman and Bloemendal, 1965; Hoagland and Askonas, 1963). The activity of these substances may vary with the physiological condition and dietary treatment of the animal.

Munro, Jackson and Korner (1964) passed cell sap through Sephadex G-25 and were able to remove the inhibitors. Bont, Razelman and Bloemendal (1965) reported that cell sap had a stabilizing effect on the structure of polyribosomes from rat liver and that passing cell sap through Sephadex G-50 did not remove it. Precipitated p^H 5 enzymes also contained the stabilizing effect. They suggested that the substance might be a ribonuclease inhibitor. Ribosomes do contain ribonuclease (see Korner, 1964, p. 210). Sidransky, Staehelin and Verney (1964) also noted that although microsomes appeared to be the primary fraction responsible for increased C^{14}-amino acid incorporation in the livers of rats force-fed a threonine-devoid diet, the cell sap also had some effect on the rate of synthesis. They attributed the difference to the amount of enzyme protein present per milliliter of cell sap. Mariani, Spadoni and Tomassi (1963) had previously indicated that the specific activity of liver amino acid activating enzymes increased during protein depletion. Stephen

(1968) reported a similar increment in rats fed a 6 per cent casein diet.

Once again, if the lysine deficiency resulted in the loss of an inhibitor or increase in activating enzymes in accord with the foregoing comments, the observed results could be explained. The increased inhibitor or decreased enzyme content of the control cell sap could account for the decreased activity of the C-T over the T-T system.

Hoagland and Askonas (1963) found a difference in the stimulatory effect of another compound (factor X) which they sedimented from the cell sap of regenerating and normal rat livers by spinning the cell sap at 100,000 x g for 12 to 13 hours. This fraction contained both protein and RNA. Some amino acid activating enzymes and s-RNA were also present in this fraction. When the X fraction was passed through Sephadex 25, 85 per cent of the RNA was removed (s-RNA) and the protein-RNA effluent (factor X) which came off had a yellowish color. This suggests that the protein part of the substance may have been transfer enzymes. Takanami (1961) reported that transfer enzymes eluted from DEAE-cellulose had a yellowish color. Hoagland and Askanas also mentioned this possibility. The effluent and the crude X fraction had the same activity. The RNA extracted from pellet X with phenol also had a stimulatory effect on protein biosynthesis but not as much as the X fraction. The phenol extracted RNA was sensitive to ribonuclease whereas the RNA in the original crude X fraction (protein-RNA complex) was quite stable. The RNA was different from s-RNA and ribosomal RNA but had

certain properties similar to m-RNA from bacteria. Hoagland and Askonas suggested that it might be a type of m-RNA that was bound to protein outside the nucleus.

It is possible that the difference in the activity of cell sap from test and control rats in our experiments was due to a difference in the X fraction from the two dietary groups. Perhaps the X fraction from the livers of amino acid deficient rats, like that from the regenerating liver, stimulated C^{14}-amino acid incorporation to a greater degree than the X fraction from the livers of control animals. This suggests a difference in transfer enzyme from test and control animals in our experiments. Perhaps a different kind of transfer enzyme is bound to the RNA in this X fraction, depending on the physiological conditions, and this in turn contributes to the control of the rate of protein biosynthesis. Protein synthesis may be partially controlled by a reaction between amino acyl s-RNA and the ribosomes (see Vogel and Vogel, 1967).

It will be recalled that in our experiments, test cell sap incubated with control microsomes did not incorporate any more leucine-C^{14} than the control system. If the X fraction in test cell sap has more activity than the control, it should increase protein synthesis. Perhaps factors in the control microsomes prevented the activity of fraction X from being demonstrated. The microsomal fraction contains, in addition to the endoplasmic reticulum and the RNP particles, many other cytoplasmic substances. Hoagland, Scornik and Pfefferkorn (1964) reported that the microsomal fraction

contributed much more to the difference in the activity of the protein synthesizing system from regenerating and normal liver than the X fraction from cell sap. Microsomes from regenerating liver was three times as active as microsomes from normal liver when incubated with a mixture containing fraction X. They (Hoagland, Scornik and Pfefferkorn, 1964) showed that normal adult rat liver microsomes contained, in excess over the amount found in regenerating microsomes, a heat-labile factor that inhibited amino acid incorporation in regenerating liver microsomes or ribosomes. It appeared to be associated with the endoplasmic reticulum and is antagonized by GTP. Gardner and Hoagland (1968) reported the presence of a unique low molecular weight RNA associated with rat liver microsomes that is not present in ribosomes. It is not known whether this unique RNA has anything to do with the inhibitors found in microsomes or not. In 1967 Hoagland and his associates (Scornik et al., 1967) reported that rat liver microsomes contain two kinds of inhibitors of amino acid incorporation in-vitro. The first is derived from lysosomes contaminating the microsomal fraction and is more prominent in normal than in regenerating liver microsomes even though lysosome content is the same. The second is a heat labile factor associated with the endoplasmic reticulum membrane that requires oxidized GSH for activity and can be reversed by sulfhydryl reagents. This reversible inhibitor interferred with the transfer of amino acids from amino-acyl s-RNA to the protein being synthesized. Perhaps this second heat labile factor is the same compound that this group of workers (Hoagland, Scornik and Pfefferkorn) reported in 1964.

In any case, it should not be a factor in our incubating mixtures since mercaptoethanol, a reducing agent, was added to all mixtures. It is possible, nevertheless, that in our studies, the activity of the inhibitor from lysosomes was greater in the casein fed rats than those fed wheat gluten. Therefore, when test enzymes were incubated with control microsomes in our study, although the test cell sap may have had more activity, it was not possible to detect if the microsomes contained much more of the inhibiting substance(s).

In order to gain more information about the mechanism controlling the increased C^{14}-amino acid incorporation by the test system, we tried to eliminate some of the above-mentioned possible variables from the protein synthesizing system by purifying the crude microsomal and cell sap preparations.

p^H 5 enzyme and microsomal fractions

The first purification step was the preparation of the p^H 5 enzyme fraction from cell sap. This eliminated some of the extraneous matter found in cell sap and facilitated better control of the quantity of enzyme protein added to the incubations. The same amount of test or control cell sap protein was used in our first incubation mixtures but this did not insure that the same amount of p^H 5 enzymes had been added. It should be recalled however, that not all stimulators and inhibitors of protein synthesis are removed by precipitating the p^H 5 enzyme fraction. Hoagland and Askonas (1963) found that factor X was still present but the amount was too small to be quantitated. Takanami (1961) found that most of the transfer enzymes were still present in the

supernatant after the p^H 5 enzyme fraction had been removed by precipitation. He isolated these enzymes by passing the supernatant (which had been readjusted to p^H 7.6) through a DEAE-cellulose column and then eluted the transfer enzymes from the column with 0.3 M KCl buffer (see Materials and Methods Section). Therefore, it is possible that a good portion of the transfer enzymes and fraction X were lost when the p^H 5 enzyme fraction was prepared. When the p^H 5 enzyme and microsomal fractions were incubated, particles from the livers of rats consuming the amino acid imbalanced diet still incorporated more leucine-C^{14} than the controls but the increment (65 per cent) was not as great (Table 6).

RNP particles and amino acid incorporation

The next purification step in our study was the treatment of the microsomal fraction with DOC in order to prepare the RNP particles free of the microsomal membranes and thus eliminate the inhibitors known to be associated with the endoplasmic reticulum. Chemical analysis of the RNP particles indicated that they were relatively free of microsomes and endoplasmic reticulum. The ratio of RNA to protein for RNP particles from both dietary groups was 0.76. Korner (1961) reported that the average ratio of RNA to protein for clean RNP particles was 0.8 (0.65 to 0.9). We found that the average ratio of RNA to RNA plus protein was 0.43, the same as Kirsch, Siekevitz and Palade (1960) reported. The particles were obtained after sedimentation for one hour at 128,000 x g in a 0.69 molar sucrose buffer. A higher molarity of sucrose

was not used since it was important for purposes of our study to sediment as many of the RNP particles as possible. Therefore, many of the organelles commonly associated with the microsomal fraction were sedimented with the RNP particles (see Korner, 1964, p. 210). The RNP particles sedimented in our study were similar to those prepared by Wettstein, Staehelin and Noll (1963) when DOC treated PMS was sedimented through 1 molar sucrose. The ribosomal pellets from the control livers were yellow and when resuspended gave a turbid solution. The ribosomal pellets from the livers of test animals were much darker, an orangy-brick red. This turbid colored solution indicates that metalloproteins--ferritin is a prominent one--may still be present (Wettstein, Staehelin and Noll, 1963). When ribosomes are sedimented through a more concentrated sucrose solution (1.5 or 1.8 molar) the metalloproteins remain as an intensely yellow band just below the interface of the bottom layer (Wettstein, Staehelin and Noll, 1963). However, when ribosomes are prepared in this manner, the lighter aggregates (monomers and dimers) are lost.

When the RNP particles were incubated with the p^H 5 enzyme fraction, it was found that the difference in the rate of protein synthesis by the fractions from the livers of rats fed wheat gluten versus those fed casein was further reduced (Table 7). However, the test system still incorporated 30 per cent more leucine-C^{14} than the control system. When the synthesizing fractions from the two dietary groups were crossed, the results were the same as those observed with cruder preparations (cell sap and microsomes). Control

enzymes incubated with test RNP particles resulted in a 21 per cent increment compared to the amount of leucine-C^{14} incorporated by the control system. Test enzymes in the control system had no effect. Increasing the ratio of enzyme protein to RNP-RNA (5.1 to 1) increased the amount of leucine-C^{14} incorporated by each system but the difference between the two systems remained the same (36 per cent) (Table 7). So once again the increased activity of the test system appears to be due primarily to the ribosomal particles with the activity of the enzyme fraction playing a lesser role.

As mentioned earlier (Leung, 1964), it is possible that there is a difference in the amount of free amino acids associated with the protein synthesizing fractions isolated from the two dietary groups. To eliminate any difference in endogenous amino acid concentration, excess amino acids (0.03 micromoles of each of the 19 free amino acids) were added to all incorporation mixtures. Adding excess amino acids caused a slight reduction in the difference between the two dietary groups but leucine-C^{14} incorporation was enhanced in both groups. These data are in agreement with Matthaei and Nirenberg (1961); Wettstein, Staehelin and Noll (1963); and Munro, Jackson and Korner (1964) who also found that the addition of an amino acid mixture enhanced C^{14}-amino acid incorporation.

When excess amounts of p^H 5 enzyme fraction and free amino acids were present in the incubation mixtures, the difference in the activity of the two dietary groups (16 per cent) was due entirely to the RNP particles (Table 8).

However, a difference in the components of the p^H 5 fractions from the two dietary groups may be masked when excess enzyme is added to the incubation mixture.

Separated enzymes, s-RNA and RNP particles

As previously indicated in this discussion, it is possible that sizable amounts of transfer enzymes as well as s-RNA may be lost in preparing the p^H 5 enzyme fraction. Therefore, enzymes and s-RNA were prepared separately according to the methods outlined in the Materials and Methods Section. It is not known whether DEAE-cellulose removes the inhibitors of protein synthesis mentioned by Munro, Jackson and Korner (1964) or the stabilizing factors reported by Bont, Rezelman and Bloemendal (1965). However, it is known that complete activity of the X fraction (Hoagland and Askonas, 1963) is retained. Also, transfer enzymes are recovered (Takanami, 1961). Separating the enzymes and s-RNA allows the addition of known amounts of s-RNA and thus eliminates one more variable from the system. It was found that the addition of one part s-RNA to five parts of RNP-RNA assured that s-RNA was not limiting in the system (Table 9). At this relative concentration, no difference between liver s-RNA from either dietary group could be detected (Table 10). The three protein synthesizing fractions from the livers of rats fed wheat gluten incorporated approximately 42 per cent more leucine-C^{14} (Tables 9 and 10). Crossing the fractions from the two systems indicated that the increased synthesis was due to a difference in both the enzyme and the

RNP fractions. The difference in the enzyme fractions from the two dietary groups could be due to a difference in the transfer enzymes, which may be the X fraction of Hoagland and Askonas (1963). Perhaps the transfer enzymes which are involved in the addition of the amino acids to the polypeptide being synthesized are "different" in some way. There may be a conformational change or the transfer enzyme-s-RNA complex may enhance protein synthesis by serving as a derepressor (see Vogel and Vogel, 1967). The exact mechanism of this enhanced activity by the enzyme fraction from the livers of rats fed wheat gluten is not known.

Also, adding the same amount of enzyme protein to each incubation does not preclude the possibility that the relative concentration among the enzymes within this fraction may be different. As explained in the Materials and Methods Section, the proteins from the two dietary groups of animals were separated electrophoretically on acrylamide gels. Conformational changes might also be identified in this manner. The electrophoretic patterns of the two sets of proteins did not disclose any discernible differences but no conclusions can be drawn without more quantitative measurement as well as specific enzyme staining.

Adding free amino acids to the incubation mixtures increased the activity of both the control and the test systems (42 and 51 per cent respectively) (Table 9). Thus it appears that chromatography removes a good portion of the free amino acids from the cell sap.

Increasing amounts of enzyme and s-RNA were incubated with a limited amount of RNP-RNA in an effort to gain more information concerning the kinetics of the two protein synthesizing systems (Table 11). A Lineweaver-Burk analysis of the data (Figure 6) indicated that the two systems had the same K_m but the test system had a higher maximum velocity (V_{max}) than the control system. That is to say, a given amount of test RNP particles incubated with a excess of amino acids and enzymes were capable of incorporating more leucine-C^{14} than the same amount of RNP particles and enzymes from the control livers. We have plotted enzyme concentration rather than substrate concentration on the abscissa. Since numerous enzymes are known to be present in the synthesizing system, K_m must be the overall dissociation constant of a complicated series of equilibria. K_m can also refer to the substrate concentration that produces half-maximal velocity and is thus a measure of the affinity between the enzyme and substrate. In our studies we have used K_m in this context. The same Michaelis-Menten constant (K_m) for the protein synthesizing systems from the two dietary groups of rats associated with the increased V_{max} for the test system, suggests an irreversible type of stimulation of enzymes and ribosomes from the livers of rats fed wheat gluten.

Accelerated protein synthesis, per mg of RNP-RNA, could be the result of more efficient use of the m-RNA present, perhaps through increased

binding of ribosomes to the m-RNA, increased rate of translation of the m-RNA strand or by accelerated release of the newly formed peptides. If more ribosomes are bound to m-RNA, it is possible that a more efficient translation is being effected i.e., the code of the m-RNA which directs the amino acid sequence in polypeptides is being read at a faster rate. Protein biosynthesis is usually thought of as being associated primarily with the polysomal fraction aggregates containing five or more ribosomes) but this is not without exception.

Sucrose Density Gradient Profiles of RNP Particles

In an effort to gain more information about the possible difference in the ribosomal fractions from the two dietary groups, the RNP particles were sedimented on sucrose density gradients (10 to 35 per cent w/v). Little difference in the size distribution of ribosomes from the two dietary groups of rats was noted except for the monomer fraction which represented 38 per cent of the RNA in the test RNP particles and 33 per cent of the control RNP particles. It should be recalled, as mentioned earlier in this section, that methods which produce ribosomal preparations containing smaller RNP particles will have proportionately more monomers and dimers than ribosomal preparations which have selectively omitted the lighter particles by sedimenting only the larger aggregates. The size distribution of the RNP particles in our studies was similar to the findings of Staehelin, Verney and Sidransky (1967) who reported that in profiles of RNP particles from fasted mice, 33 to 39 per cent of the liver ribosomal RNA was present in the monomer fraction.

It is possible that what appears as a larger monomer fraction in the test rats may be accounted for by the presence of more ferritin associated with their ribosomal fraction. It will be recalled that these ribosomes had an orangy-red color in comparison to the yellow color of the ribosomal pellets from the control animals. A yellow band, approximately one-fourth to one-third from the top of the sucrose density gradient, was consistently observed in the gradients prepared from test ribosomes. The amount of RNA present in the several gradient fractions was measured by reading the O.D. of the solution at 260 mμ. Ferritin also absorbs light at this wavelength (Drysdale and Munro, 1967). Sucrose density gradients containing horse ferritin indicated that it sedimented in the same area as the monomers (Figure 7). This then, could correspond to the "hump" on the lighter (10 per cent) side of the monomer fraction of the test RNP particles in our experiments.

In agreement with our findings, other workers have found a difference in the rate of C^{14}-amino acid incorporated by incubation mixtures containing ribosomal aggregates of the same size. Decken (1967) maintained rats for several days on a protein free diet and, 14 hours before killing, he changed part of the rats to a protein-rich diet. He found no change in the sedimentation pattern of the microsomal subfractions or the polysomes but dietary protein enhanced the ability to incorporate C^{14}-amino acids. Decken suggested that the ribosomes had different read-out velocities. That is to say, the m-RNA, which codes for polypeptide synthesis, moved along the ribosomes (was translated by the ribosomes) at a faster rate. Sox and Hoagland (1966)

found that, per mg RNA, heavy polysomes from the liver of refed rats incorporated more C^{14}-amino acid than polysomes of the same size from starved animals.

Other reports in the literature with findings similar to ours suggest that protein synthesis may be controlled by regulatory mechanisms within the RNP particles themselves. All of the following studies involved altered polysome function apparently independent of m-RNA synthesis. This suggests cytoplasmic control of protein synthesis which involves translation of the code carried by pre-existing m-RNA rather than the transcription of nuclear DNA necessitating the synthesis of new m-RNA. Polysomes extracted from tissue cultures of chick embryo cells starved for calf serum remained intact and capable of immediate function in-vivo and in-vitro when serum was added (Soeiro and Amos, 1966). Trypsin has been reported to activate ribosomes obtained from sea urchin eggs (Monroy, Maggio and Rinaldi, 1965). Also, Salb and Marcus (1965) found that ribosomes from HeLa cells in mitosis, which exhibit low activity, could be activated by trypsin. Dietz, Reid and Simpson (1965) reported that polysomes from yeast were more active, per mg of ribosomal RNA, during growth. Specific repression of synthesis of a protein necessary for steroidogenesis in rats likewise seems to occur through an effect on translation. The synthesis of apoferritin in response to the administration of iron was independent of additional m-RNA synthesis (Drysdale and Munro, 1966). Other workers have also suggested that a cytoplasmic control mechanism, involving a small metabolite (perhaps a peptide) may regulate protein synthesis at the ribosomal level (see Vogel and

Vogel, 1967, p. 531). It has been suggested that these molecules may act as inducers or repressors. Perhaps the lysine rich nucleoproteins in the ribosomes play a role in regulating protein biosynthesis at the cytoplasmic level similar to the controlling role proposed for the lysine rich histones at the transcriptional level in the nucleus.

It is not known whether all of the fractions from the test sucrose gradient in our study incorporate more leucine-C^{14} than the corresponding fraction from the gradient prepared from control RNP. It is possible that the difference in rate of C^{14}-amino acid incorporation is restricted to RNP particles of a given size. Sidransky, Staehelin and Verney (1964) calculated that the increased C^{14}-amino acid incorporation observed in threonine-devoid rats was due primarily to a difference in the rate of incorporation, per mg RNA, by the smaller particles. Decken (1967) found that all fractions from the sucrose gradient of rats fed a protein containing diet incorporated more C^{14}-amino acids, per mg RNA, than the corresponding fraction from the animals fed a protein free diet. It thus appears that the comparative activity of the different sucrose gradient fractions may vary with the previous dietary conditions.

All amino acid deficient diets may not have the same effect on the ribosomal sucrose gradient profile and C^{14}-amino acid incorporation. We found in this study that the lysine deficient diet caused increased C^{14}-amino acid incorporation without a change in the profile. Sidransky, Staehelin and Verney (1964) found that force-feeding a threonine-devoid diet to rats for 3

days resulted in increased amino acid incorporation and the sucrose gradient profile contained proportionately more heavy polysomes than rats fed the same amount of a diet containing the complete amino acid mix. A single feeding of a tryptophan deficient mix caused a shift of the polysomes to lighter aggregates, particularly monosomes, and less amino acid incorporation (Wunner, Bell and Munro, 1966). When compared with controls fed a complete amino acid mix, feeding methionine or isoleucine-devoid amino acid diets caused the ribosome profile to shift toward heavier aggregates with fewer monosomes. C^{14}-amino acid incorporation increased 49 and 28 per cent respectively. Wunner (1967) reported that diets devoid of arginine, phenylalanine or methionine-cystine did not materially affect the liver polysome profile.

Nature of the Protein Synthesized

In our studies, which involved a lysine deficient diet, leucine-C^{14} incorporation was used as a measure of protein synthesis. The increased uptake of amino acids in our test system appears to be a generalized phenomenon. When we used alanine, phenylalanine, and lysine for uptake studies, we found that each had an increased uptake distinctive to itself (Tables 12 and 13). Furthermore, when the relative incorporation of these amino acids was examined, it was found that it was the same in both the test and control experiments. Thus, it is likely that the peptides being synthesized may be similar in the two systems. However, to be more certain of this, simultaneous uptake experiments would have to be carried out. Our data indicate that the increased

rate of C^{14}-amino acid incorporation by the test system is a general phenomenon and not just an artifact such as miscoding for one amino acid. Thus it would appear that there is a general increase in the rate of protein synthesis.

Hepatic Injury and Enhanced Protein Biosynthesis

It is possible that the enhanced liver protein synthesis observed in rats fed an amino acid deficient diet may be a common reaction in response to injurious agents. Prolonged consumption of amino acid imbalanced diets may cause hepatic injury. Rats fed amino acid deficient diets for prolonged periods of time incorporated more labelled amino acids (Stirpe and Schwarz, 1963; Nimni and Bavetta, 1961). Corless and Gray (1967) reported increased liver cell-free protein synthesis in rats which had been exposed to lethal X-irradiation. Enhanced incorporation of amino acids into liver protein has been found in studies with other agents producing hepatic injury (see Sidransky, Staehelin and Verney, 1964). Drugs, such as 3-methyl cholanthrene and phenobarbital cause increased C^{14}-amino acid incorporation (Gelboin and Sokoloff, 1961; Decken and Hultin, 1960). Drabkin (1963) and Hoagland and Askonas (1963) found that regenerating liver after partial hepatectomy incorporated labelled amino acids at a much faster rate. The administration of iron, a toxic substance, causes increased liver protein biosynthesis (Drysdale and Munro, 1966). The observations of Gray and French (1962), Lust (1966) and Williams, Ganoza and Lipmann (1965) suggest that protein synthesis is increased during infection. Lust found that bacterial infection with Diplococcus pneumoniae, type I

Strain A_5 caused an elevation of rat liver and small intestine protein biosynthesis. On the other hand a decreased rate of synthesis was observed in muscle. Administration of adrenal glucocorticoid hormones gave a pattern similar to that of the bacterial infection itself. Similarly, Guidotti, Rossi and Ragnotti (1963) found that Salmonella typhimurium toxin caused an increase in the rate of C^{14}-glycine incorporation in liver slices. Young, Chen and Newberne (1968) also found that infection with Salmonella typhimurium reduced the uptake of radioactivity by muscle ribosomes and resulted in lowered levels of heavy polyribosomes and increased proportions of the light ribosome species.

Hormonal Control of Protein Biosynthesis

Korner (1964, p. 213) stated that "the ability of ribosomes of animal origin to incorporate amino acids into protein is under hormonal control." In our study, we have shown that the ribosomal fraction isolated from the liver of test animals contributes to the increased incorporation of C^{14}-amino acids. Korner (1960) observed that microsomal fractions isolated from the livers of cortisone-treated rats have an enhanced capacity for amino acid incorporation. In our study, food intake was depressed. This would favor the production of catabolic hormones having as part of their action, increased protein synthesis in the liver and concomitant loss of protein from the carcass (see Munro, 1964, p. 449). Amino acid imbalanced diets could also favor the production of catabolic hormones in order to increase available amino acids

for incorporation into liver protein. It may be recalled that Castellanos and Arroyave (1961) found increased excretion of 17-hydroxycorticosteroid hormone in marasmus and a decrease in children suffering from kwashiorkor. Although the hormonal response in our study is not known, it is possible that hormones may play a significant role in controlling the observed changes.

SUMMARY

The effects of an essential amino acid (lysine) deficient diet on the mechanism of protein biosynthesis in rat liver was investigated. Rats fed a 20 per cent wheat gluten diet (test) consumed less food per day (10 versus 14 grams and gained less weight (38 versus 155 grams) than controls fed a 20 per cent casein diet. The average feed efficiency ratios of the two dietary groups were 0.15 and 0.36 for the test and control animals respectively. The average liver weight was the same for the two dietary groups. The livers from the rats fed an amino acid deficient protein were pale, yellowish and friable. Microsomes and ribosomes from the amino acid deficient rats appeared to contain more metallo-proteins.

When equal amounts of cell sap and microsomal protein from fasted rats were incubated, the preparations from the rats fed wheat gluten incorporated 158 per cent more leucine-C^{14} than those from rats fed casein. Crossing the protein synthesizing fractions indicated that the microsomal fraction was primarily responsible for the increased activity.

Equal amounts of microsomal and p^H 5 enzyme fractions from test animals continued to incorporate more leucine-C^{14} than preparation from the controls.

The ratio of RNA to protein of RNP particles prepared from the microsomal fractions was the same for the two dietary groups (0.76). RNP particles and p^H 5 enzymes from the livers of test animals continued to incorporate more leucine-C^{14} than the controls, regardless of the enzyme concentration. The addition of free amino acids to incubation mixtures containing excess p^H 5 enzymes increased the specific activity of the test system 5 per cent and the control system, 16 per cent. Under these conditions, there was no stimulation of amino acid incorporation by the test enzymes.

Purified activating and transfer enzyme protein (1 mg), s-RNA (0.2 mg) and RNP particles (1 mg RNA) from the amino acid deficient rats incorporated 42 per cent more leucine-C^{14} than the control system. Enzyme and RNP particles contributed equally to the increased specific activity of the test system. Adding free amino acids increased the specific activity of the test system 51 per cent and the control system, 42 per cent.

Regardless of the C^{14}-amino acid used (phenylalanine, alanine, leucine or lysine) the reactions containing particles from amino acid deficient rats had a higher specific activity than the controls.

Increasing concentrations of enzyme and s-RNA were incubated with a constant amount of RNP-RNA. A Lineweaver-Burk analysis indicated that the K_m was the same for the two dietary groups but the V_{max} for the wheat gluten group was greater. This would indicate an irreversible activation of the test system.

No difference was observed in the protein electrophoretic patterns of the purified protein synthesizing enzymes from the livers of the two dietary groups. Sucrose density gradient profiles of the RNP particles were similar except for a larger monomer peak which might be attributed to the increased concentration of metallo-proteins in the particles from rats fed wheat gluten.

CONCLUSION

The results of this investigation indicate that feeding weanling rats a diet containing 20 per cent wheat gluten (a poor quality protein lacking primarily lysine) for 33 days causes an increase in the rate of liver protein synthesis as measured by C^{14}-amino acid incorporation in-vitro. Since the uptake of several labelled amino acids was similar, it is concluded that the increased activity is a general phenomenon and suggests that similar polypeptides are being synthesized by the two systems. Lineweaver-Burk analysis indicate that the increased stimulation is irreversible.

Both the ribosomal and enzyme fractions of the purified system contributed to the increased specific activity. In the crude preparations, the fraction containing the enzymes and s-RNA did not contribute to the increased activity. It is postulated that certain inhibitors may be present or a loss of transfer enzymes may occur that could prevent the activity from being manifested. No change was observed in the sedimentation pattern of the RNP particles. Therefore it is concluded that the difference in the activity of the ribosomal fraction is at least in part an intrinsic property of the RNP particles themselves. Enzymes eluted from a DEAE-cellulose column had a greater effect on C^{14}-amino acid incorporation than other enzyme preparations. The column effuent contains proportionately more transfer enzymes than p^{H} 5 enzyme

precipitates. Therefore it appears that the difference in the activity of the enzyme fraction may be due to a change in the transfer enzymes.

As applied to the problem of chronic malnutrition, it therefore seems possible that in the intact animal there occurs an irreversible increase in liver protein biosynthesis. This may develop at the expense of other proteins. The change may come about in an effort to meet the increased protein demand which however, cannot be achieved because of the amino acid deficiency.

APPENDICES

Supplemental Data

Appendix 1

Amino acid content of rat diets compared with the recommended minimum required amounts

Component	Recommended[a] for growth % of diet	Milk casein[b] protein % of diet	Wheat gluten[c] protein % of diet
Crude protein[d]	20	20	20
Net protein[e]	12		
Net amino acids			
L-tryptophan	0.15	0.27	0.21
L-histidine	0.30	0.60	0.46
L-lysine	0.90	1.60	0.38
L-leucine	0.80	2.01	1.50
L-isoleucine	0.50	1.31	0.92
L-phenylalanine	0.90[f]	1.08	1.09
L-methionine	0.60[g]	0.62	0.35
L-cystine		0.08	0.43
Total sulfur amino acids		0.70	0.78
L-threonine	0.50	0.86	0.53
L-valine	0.70	1.48	0.95
L-arginine	0.20	0.81	0.87
Non essential amino acids	6.45		

a. Animal Nutrition Committee-Subcommittee on Laboratory Animals, 1962. Nutrient Requirements of Domestic Animals, No. X (Nutrient Requirements of Laboratory Animals), Pub. 990, Nat'l. Acad. Sci.-Nat'l. Res. Council, Wash., D. C.

b. Orr, M. L., and B. K. Watt 1966 Amino Acid Content of Foods. Home Econ. Res. Report #4, USDA. Supt. of Documents, U.S. Govt. Printing Office, Wash. D. C., p. 48.

c. Ibid., p. 30. Computed on the basis of 5.70 g protein/g nitrogen as stated on p. 58.

d. Dietary N X 6.25.

e. Dietary N X 6.25 with a true digestibility and a biological value of 100 per cent.

f. One-third may be supplied as L-tyrosine.

g. One-third to one-half may be supplied by L-cystine.

Appendix 2

Complete vitamin diet fortification mixture

	g/100 lbs. diet
Vitamin A concentrate (200,000 units/g)	4.5
Vitamin D concentrate (400,000 units/g)	0.25
Alpha Tocopherol	5.0
Ascorbic Acid	45.0
Inositol	5.0
Choline Chloride	75.0
Menadione	2.25
p Aminobenzoic Acid	5.0
Niacin	4.5
Riboflavin	1.0
Pyridoxine Hydrochloride	1.0
Thiamine Hydrochloride	1.0
Calcium Pantothenate	3.0
	mg/100 lbs. diet
Biotin	20
Folic acid	90.0
Vitamin B-12	1.35

Appendix 3

Buffers

Component	MN Buffer[a] M/liter	K Medium A[b] M/liter	K Medium B[c] M/liter	Modified[c] K Medium A M/liter
Sucrose	0.25	0.35	0.90	0.25
KCl	0.06	0.025	0.025	0.025
$MgAc_2$	0.01	----	-----	-----
$MgCl_2$	---	0.004	0.004	0.004
Tris-p^H 7.6	0.01	0.05	---	0.05
Mercaptoethanol	0.006	---	---	---

a. Buffer used by Matthaei and Nirenberg (1961).

b. Buffer used by Kirsch, Siekevitz & Palade (1960).

c. A modification of the buffer used by Kirsch, Siekevitz & Palade (1960).

Appendix 4

Description and source of materials used

Material	Description	Source
Animals, rats	Sprague-Dawley	Charles River Breeding Labs., Wilmington, Mass.
Chemicals[a]		
L-Amino acids		
Non-radioactive		
Alanine		Calbiochem
Arginine-HCl		Sigma Chemical Co.
Asparagine. H_2O		"
Aspartic		Calbiochem
Cysteine HCl, hydrate		"
Glutamine		"
Glutamic		"
Glycine		"
Histidine. HCl, monohydrate		Nutritional Biochemicals
Isoleucine		Calbiochem
Leucine		"
Lysine, Mono HCl		Sigma Chemical Co.
Methionine		Calbiochem
Proline		"
Phenylalanine		"

Appendix 4 (Continued)

Material	Description	Source
Serine		Calbiochem
Threonine		"
Tryptophan		"
Tyrosine		"
Valine		"
Radioactive		
UL C^{14}-Alanine	Specific activity: 369mc/mmole (0.5μc/0.004 μmole)	New England Nuclear
UL C^{14}-Leucine	Specific activity: 251mc/mmole (0.5μc/0.002 μmole)	"
UL C^{14}-Lysine	Specific activity: 227 mc/mmole (0.5μc/0.0022 μmole)	"
UL C^{14}-Phenyl-alanine	Specific activity: 379mc/mmole (0.5μc/0.0014 μmole)	"
GL-H^3-Phenyl-alanine	Specific activity: 2.8c/mmole 2.0μc/ .0007 μmole)	"
ATP, crystalline	Di sodium salt	Nutritional Biochemicals
CTP	Sodium salt, Type IV	Sigma Chemical Co.
DEAE-cellulose	Lot No. 7392	Eastman Organic Chem. Rochester, N. Y.

Appendix 4 (Continued)

Material	Description	Source
DOC	Sodium salt	Sigma Chemical Co.
GTP	Sodium salt, Grade II-S	"
Labtrol	70 mg/ml	Dade Reagent Inc., Miami, Florida
2-Mercaptoethanol	Anhydrous Type I	Sigma Chemical Co.
Naphthalene		Eastman Kodak
PEP	Trisodium salt hydrate	"
Phenol	Reagent grade	Fisher
POPOP	Scintillation Grade	Nuclear Chicago
PPO	"	"
Pyruvate Kinase	Type II from rabbit skeletal muscle. Crystalline suspension in $(NH_4)_2SO_4$ various lots containing 4-9 mg/ml which converted 400-625 μmoles PEP/min.	Sigma Chemical Co.
Tris buffer	"Sigma 7-9" Primary Std and Biochemical Buffer	"
UTP	Trisodium salt, Type IV	"

Appendix 4 (Continued)

Material	Description	Source
Diets containing		
Wheat gluten protein	Control No. 3576	Nutritional Biochemicals Corp., Cleveland, Ohio
Casein protein	Control No. 3391 See Table 1, in the text	"
Supplies		
Columns, chromatographic	Pyrex brand 400 x 10 mn (inside) w/coarse porosity fretted disc 14/35 joint	Thomas & Co.

a. All other chemicals not listed were Standard Reagent Grade.

Appendix 5

Growth of two groups of rats on experimental diets for varying lengths of time

Controls

Group	I^a	I^a	II^a	II^a
Experiment	1	2	3	4
On diet (days)	48	55	40	53
Wt. beginning (g)	55	45	40	42
Wt. end (g)	281	264	216	254
Body wt. gained (g)	226	218	176	212
% Body wt. gain	409	486	450	506
Liver wt (g)	6.5	6.4	6.0	6.2
Liver/100g body wt. (g)	2.3	2.4	2.8	2.4

Test

Group	I^b	I^b	II^b	II^b
Experiment	1	2	3	4
On diet (days)	48	55	40	53
Wt. beginning (g)	56	45	40	40
Wt. end (g)	140	117	85	99
Body wt. gained (g)	84	72	45	59
% Body wt. gain	151	160	113	148
Liver wt. (g)	3.2	3.0	2.7	2.5
Liver/100g body wt. (g)	2.3	2.6	3.1	2.6

Appendix 5 (Continued)

	Ratio $\frac{\text{Test}}{\text{Control}}$			
Group	I	I	II	II
Experiment	1	2	3	4
Wt. gain @ 33 days	0.34	0.29	0.24	0.24
Wt. gain @ termination of experiment	0.37	0.33	0.26	0.28
Per cent gain	0.37	0.33	0.26	0.29
Liver/100g body wt. (g)	0.99	1.06	1.13	1.05

a. Mean values of 2 rats.

b. Mean values of 4 rats.

Appendix 6

A comparison of the incorporation of 4 different radioactive amino acids into liver proteins by particles from rats fed wheat gluten or casein

	CPM per mg RNP-RNA			
	Control		Test	
Amino acids	Duplicates	Average	Duplicates	Average
	Experiment 8			
Leucine-C^{14}	4027/4144	4086	5059/5322	5190
Lysine-C^{14}	2619/2711	2665	3109/3138	3124
Alanine-C^{14}	3878/3915	3896	4818/5345	5082
Phenylala-H^3	2415/2198	2262	2564/3115	2840
Leucine-C^{14}*	134	134		
	Experiment 9			
Leucine-C^{14}	3739/3712	3726	4090/4420	4255
Lysine-C^{14}	2723/2812	2768	3696/3456	3576
Alanine-C^{14}	3650/3715	3684	5313/5184	5248
Phenyala-C^{14}	2049/2104	2077	2765/2898	2832
Leucine-C^{14}*	109	109		
	Experiment 11			
Leucine-C^{14}	4424/4056	4240	5600/5880	5740
Lysine-C^{14}	3144/3056	3100	4312/4160	4236
Alanine-C^{14}	3792/3736	3764	5552/5288	5420
Phenyala-C^{14}	2320/2200	2260	2872/2960	2916
Leucine-C^{14}*	340	340		

All reactions contained the following in μ moles: contained 65 KCl, 70 sucrose and 0.2 GTP. Experiments 8 and 9, 1 mg enzyme protein, 0.2 mg s-RNA and 1 mg RNP-RNA; Experiment 11 contained 0.5 mg, 0.1 mg and 0.5 mg respectively.

*Zero time incubation.

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