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EFFECTS OF DIETARY PROTEIN QUALITY ON HEPATIC XANTHINE OXIDASE ACTIVITY AND URINARY INORGANIC SULPHUR EXCRETION IN THE RAT

by

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A Thesis Submitted to the Faculty
of Agricultural Sciences in Partial Fulfillment of
The Requirements for the Degree of

MASTER OF SCIENCE IN AGRICULTURE

Major: Food Technology and Nutrition

Minor: Chemistry

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LIVER XANTHINE OXIDASE & URINE S

Angel

ACKNOWLEDGEMENTS

The author wishes to extend his thanks to Dr. R. Tannous for his guidance, advice, suggestions and stimulating discussions during the course of this study.

Acknowledgements are due to all the staff of the Division of Food Technology and Nutrition, for their cooperation and encouragement.

The author is indebted to Mr. V. Djibelian, for his valuable assistance in the xanthine oxidase assay; and to Mr. S. Checkijian for his assistance in the xanthine oxidase assay and for drawing the graphs.

Thanks are also extended to Miss Arpi Unkababian for typing this manuscript.

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ABSTRACT

The effects of three dietary protein sources and amino acid supplementation on four methods of evaluating dietary protein quality were investigated in the rat. The dietary protein sources used were casein, Laubina 103 (a mixture of burghol, chick-peas and dry skim milk) and "B & L" (a mixture of burghol, lentils and dry skim milk).

Both the activity of liver xanthine oxidase per gramme of liver nitrogen and urinary inorganic sulphur-to-nitrogen ratio were significantly increased by the addition of methionine to the diet. The addition of lysine or threonine had no significant effect.

The only significant difference in protein efficiency ratio values of the three diets used was that of casein being significantly higher than that of "B & L".

Net protein utilization values for the three diets were significantly different from each other. The values were in the following descending order: casein, Laubina 103 and "B & L".

Protein efficiency ratio and net protein utilization values were found satisfactory for evaluating protein quality in general. However, liver xanthine oxidase and urinary inorganic sulphur were found to be particularly affected by dietary intake of methionine.

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INTRODUCTION

Animal organisms require proteins in their diets to synthesize and maintain their tissue proteins. The primary function of dietary proteins is to provide a mixture of amino acids, in the proper proportions required by the animal organism. All methods of evaluating the nutritive value of proteins must, therefore, evaluate this function, directly or indirectly (1). Thus, a protein is considered of good quality if it provides the organism with the essential amino acids in sufficient quantities and in proper proportions.

Because of the prevalence of protein malnutrition in developing countries (2), the need for evaluating the various proteins in the different foodstuffs and diets of these countries becomes imperative. It is also important that some correlation should exist among the various procedures employed in evaluating protein quality.

Several methods of evaluating the nutritive value of proteins have been devised. The method of Mitchell (3), based on measurement of nitrogen loss or retention by animals, is considered by many workers to give the most reliable results (4). This method is quite laborious, however, and several attempts, based on the effects of dietary proteins on blood and tissue composition and

growth rates of young animals were also devised. Other attempts were made to correlate the essential amino acid contents of proteins, determined by chemical and microbiological methods, with their nutritive values. The methodology of protein evaluation has been reviewed extensively (1,5-8), and a recently published review by Campbell (4) includes critical appraisal of the different methods of protein evaluation, presenting a discussion of the relative advantages and drawbacks of each.

Under certain conditions, liver protein content is affected by the quality and quantity of dietary protein. This is based on the fact that liver plays an important role in the dynamic state of tissue proteins (1). Consequently, it is possible that changes in liver protein content will represent changes in liver enzyme protein content. More than thirty enzyme systems were found to decrease in total activity, to varying degrees by protein depletion (1). Of these enzymes, xanthine oxidase was the most susceptible, and a correlation between its activity and the nutritive value of proteins was investigated (9-15).

Another method of studying the nutritive value of proteins was proposed by Miller and Naismith (57). These authors observed that many human diets tend to be limited in their sulphur-containing amino acid content. Thus, they suggested that the sulphur-to-nitrogen ratio of diets may be considered as an index of protein quality (58).

This ratio was found to be in agreement with the ratio of inorganic sulphur to nitrogen in urine (5). Considering the urinary nitrogen-to-creatinine ratio as an index of the quantity of protein ingested, the product of the two ratios, namely, the ratio of inorganic sulphur to creatinine, was proposed as an index of both the quality and the quantity of dietary protein.

The purpose of the present investigation is to study the effects of dietary proteins and amino acid supplementation on liver xanthine oxidase activity and urinary inorganic sulphur excretion in the rat. It was of particular interest to study the urinary sulphur excretion by the rat in more detail, since all reported investigations on its relation to protein nutrition describe studies conducted on humans. Another reason for chosing these two methods of protein evaluation was that both the excretion of inorganic sulphur in urine and the activity of liver xanthine oxidase are largely affected by the amount of sulphur-containing amino acids, particularly methionine in case of xanthine oxidase, in the diet. An attempt to compare the results with those obtained by other methods of protein evaluation was also made.

REVIEW OF LITERATURE

I. Liver Xanthine Oxidase

Liver Proteins:

Liver proteins, like all tissue proteins, contribute to the over-all dynamic state of body proteins (1). Addis *et al.* (16-19) were the first to emphasize that the protein content of liver, under certain experimental conditions, is a function of both the quality and the quantity of dietary protein. Kosterlitz and Campbell (20-22) developed several methods for estimating labile liver proteins of rats, following a brief fasting period, and used these values as a basis for a method of evaluating dietary proteins. These methods, however, were not found to be sensitive to certain amino acid deficiencies.

Liver Enzymes:

It is conceivable that changes in tissue protein content may represent changes in enzyme protein content of these tissues. The possibility of using liver enzyme activities in estimating the nutritive value of proteins was initiated by the work of Miller (9) in 1948. This author found that inanition caused losses in the activities of hepatic catalase, alkaline phosphatase, xanthine oxidase and cathepsin. The losses observed were in correlation with losses in liver protein content. More than thirty

enzymes were reported to decrease in total activity with body protein depletion (1). Several workers (9,23-26) pointed out that the activities of some enzymes were reduced more rapidly than liver proteins, while some other enzymes increased in their activities due to protein depletion. Of all the hepatic enzymes studied, xanthine oxidase was the most labile.

Liver Xanthine Oxidase:

The presence of xanthine oxidase activity in animal tissues was reported in the latter part of the nineteenth century. The distribution of the enzyme in the various organs of different animal species has been studied by several workers (27) and very recently by al-Khalidi *et al.* (28). Xanthine oxidase activity is present in mammalian milk (27) and even in some bacteria (29).

Xanthine oxidase is a flavoprotein, the prosthetic group of which contains iron, molybdenum and flavine adenine dinucleotide (27). The enzyme possesses two types of activity. It may act as an oxidase, passing electrons to oxygen, or it may pass electrons to other electron acceptors such as ferricytochrome c, thus acting as a dehydrogenase (30). The enzyme catalyzes the conversion of the substrate, xanthine and hypoxanthine, into uric acid. The oxidation of at least ten purines, and more than thirty aldehydes, is catalyzed, at varying rates, by the enzyme (31).

The activity of liver xanthine oxidase is extremely labile, and is affected by several factors. Reduction in rat liver xanthine oxidase activity was observed when dietary riboflavin was less (32,33) or more (33) than its requirement. Williams _et al_. (34) reported reduction in the activity of the enzyme in vitamin B_{12}-deficient rats. It was postulated that this effect is an indirect one, through the general effect of the vitamin on protein metabolism. Similarly, folic acid was reported to affect the activity of this enzyme, probably due to the participation of folic acid in methyl group synthesis (35). Molybdenum (36) and iron (37) were found necessary for maintaining normal levels of xanthine oxidase activity. Reinhold _et al_. (38) are investigating the effects, if any, of zinc deficiency on rat liver xanthine oxidase activity. Cox and Harris (39) observed reduction in the activity of the enzyme in the livers of rats fed diets containing excess of zinc.

As early as 1945, McQuarrie and Venosa (40) demonstrated that different levels of dietary protein, as well as different protein sources, caused profound differences in xanthine oxidase levels in rat livers. A high correlation of the enzyme activity with the nutritive value of protein was established by Williams, Elvehjem, Litwack and associates (11-14,34,41). The very labile xanthine oxidase, they believed, might correlate better than other

enzymes of the liver with the over-all metabolism of body proteins. Furthermore, they pointed out that the enzyme is more susceptible to subtle changes in the quality of dietary protein than growth, and could, therefore, be considered a better index of quality, since growth is the resultant of other components of the body, in addition to tissue protein gains (14).

The fact that certain proteins may, more drastically, be affected by a simple amino acid deficiency than other tissue proteins was emphasized by many workers. Xanthine oxidase activity was shown to be particularly susceptible to methionine deficiency (10,41-43). The enzyme was reported to be sensitive to tryptophan deficiency also, but to a lesser extent (41). Similarly, diets deficient in lysine (43-45) and isoleucine (46) failed in maintaining the normal level of enzyme activity in rat livers. Histidine deficiency had little effect in this respect (43, 47).

The effects of supplementing different amino acids to diets of rats on liver xanthine oxidase activity were also investigated. Methionine supplementation to the diet increased the activity of the enzyme (48-50), and could maintain it in rats kept on low-protein diets (51). Tamura <u>et al</u>. (49) studied the effects of supplementing diets with methionine, threonine, lysine and phenylalanine on the activity of the enzyme in the livers of rats kept on 9 percent

casein diet. The activity was increased by methionine and threonine, but reduced by lysine and phenylalanine. Litwack _et al_. (52) studied the effects of essential and non-essential amino acids on the activity of the enzyme. They found that both the essential and non-essential amino acids increased the levels of enzyme activity, as well as the growth of rats. They also reported that nitrogen sources other than amino acids, such as ammonium citrate and urea, maintained adequately the enzyme activity. These authors suggested that ammonium and urea nitrogen could contribute to the maintenance of the enzyme activity, possibly through their participation in the synthesis of non-essential amino acids, or by sparing the amino acids already present in tissues and are available for enzyme protein synthesis.

It is important to emphasize that the loss of enzyme activity in the livers of rats fed protein-free diets, or inane rats, is primarily due to loss of enzyme protein, and not due to the accummulation of inhibitors or loss of cofactors (11).

It is interesting to note that the activity of the enzyme in rat liver increases with age. Rats are born without detectable xanthine oxidase activity in their livers (53); the activity then increases in the 21 days old weanling rat to one-fourth to one-half the level observed in the adult (54, 55).

The methods used by different authors for evaluating protein quality, employing liver xanthine oxidase response, vary considerably. Litwack _et al._ (12) fed adult rats 5,10,18 and 30 per cent protein from each of casein, lactalbumin, gliadin and beef round, after a priliminary 7-day adjustment period on a stock diet. A plateau in liver xanthine oxidase activity, indicating a state of equilibrium, was reached nine to ten days after the experimental diets were applied. The values obtained for liver xanthine oxidase activities were then plotted vs. levels of protein in the diet, for each protein fed, and the slope of the curve was considered as the enzyme response index. Enzyme response indices were expressed in terms of gliadin, whose response was considered by these authors as unity. In a subsequent study, Litwack and co-workers (13) omitted the 30 percent protein level, since its enzyme response value fell on a plateau, and reported that their results were in agreement with the results of growth experiments carried on weanling rats. Dju _et al._ (15) devised several methods for assaying the quality of milk proteins by measurement of xanthine oxidase response in livers of adult rats fed the diets for an experimental period of nine days. They expressed their results in different ways, in an attempt to assess a correlation between the activity of the enzyme and the nutritive values of different proteins; and compared their results with

those of other methods of protein evaluation. A method for studying nitrogen requirement of rat, using liver xanthine oxidase activity response, was recently described by Mezencesco and Popescu-Stefanesco (56). Liener and Wada (48) used xanthine oxidase response to measure the availability of methionine from soya bean meals, in six-week feeding trials.

Adult rats, at various ages, were usually used in xanthine oxidase studies. Litwack et al. (14), however, stressed that weanling rats should be studied. This permits, they suggested, the study of the animals while they are in a generative state with regard to tissue proteins.

II. Urinary Inorganic Sulphur

Dietary Sulphur:

The possibility of correlating the sulphur content of human diets with protein quality was first investigated by Miller and Naismith (57) in 1958. By inspecting the patterns of many human diets, they found that the sulphur-containing amino acids tend to be either the limiting, or the second-limiting amino acids in the diet. Consequently, they suggested that the total sulphur content of mixed dietaries might provide a simple chemical means for evaluating protein nurture. A correlation was reported by these authors, between the sulphur content of the diets studied and the "net dietary-protein value" (i.e., biological value

X digestibility X percent crude protein = net protein utilization X percent crude protein). Miller and Donoso (58) proposed using the sulphur-to-nitrogen ratio in diets as a direct quality index score, from which the "net dietary-protein value" could be calculated, by the aid of the nomograph of Miller and Payne (59). From studies on 25 human diets, mainly from Africa, methionine supplementation could improve the net protein utilization (NPU) values by more than 5 units (58). The "net dietary-protein value" was calculated from the ratio $\frac{S\ \%}{N\ \%}$ X 1000, and the values were found to be in good agreement with rat assays.

Urinary Sulphur:

The end product of the metabolism of protein sulphur (contained mainly in cystine, cysteine and methionine) is inorganic sulphate, which is excreted in the urine. Preformed inorganic sulphate and other forms of sulphur in the diet add to the urinary inorganic sulphate excretion (60). Other forms of sulphur present in urine are the organic and the esterified sulphates (61). However, the inorganic sulphate accounts for about 90 percent of total sulphur excretion in human urine (5).

Friedel (62) studied nitrogen and sulphur excretion in nutritionally disturbed infants. He found higher nitrogen-to-sulphur ratios in severely malnourished infants than normal ones, and considered a low nitrogen-to-sulphur ratio as a sign of regenerative metabolism after acute

nutritional disturbance. A high correlation between urinary and faecal sulphur and nitrogen excretion, on one hand, and nitrogen and sulphur retention, on the other hand, was reported in girls at low levels of nitrogen intake (63). It was found, in a study with adult volunteers, that the sulphur-to-nitrogen ratio in the diet was in agreement with the ratio of inorganic sulphur to nitrogen in urine, and that both ratios reflected the quality of dietary protein (5).

Powell et al. (64) suggested that the urinary nitrogen-to-creatinine ratio could be taken as an index of the quantity of protein ingested. This was based on the fact that creatinine excretion is fairly constant for the same individual, which makes it a suitable reference value for evaluating any alterations in urinary nitrogen excretion.

Since the urinary inorganic sulphur-to-nitrogen ratio may be taken as an index of protein quality when sulphur amino acids tend to be the limiting ones, and the urinary nitrogen-to-creatinine ratio may be considered as an index of the quantity of ingested protein, then the product of the two ratios, namely, the inorganic sulphur-to-creatinine ratio, may be considered as a combined index of both the quality and the quantity of protein consumed (64, 65).

All the studies that have been reported so far in

this connection were carried on human subjects. The method has been suggested as an easy one, applicable in nutrition surveys.

III. Other Methods of Protein Evaluation

Other methods of protein evaluation employed in the present investigation include growth, protein efficiency ratio (PER) and net protein utilization (NPU) assays. These methods have been extensively reviewed (1,4,5-8) and a recent review was presented by Vessal (66). A brief description of each of these assays will be presented in this discussion.

Growth:

The rate of growth of animals provides a simple way of evaluating dietary proteins. Growth of animals on a diet deficient in one or more of the essential amino acids will be depressed to varying degrees, depending on the degree of deficiency. Dietary factors, other than proteins, will also play a role in determining the total growth of an animal. However, when these factors are kept at a minimum, growth will provide an over-all index of dietary protein quality (4).

Hegsted *et al.* (67) have pointed that the metabolic utilization of dietary proteins in man and the rat have close resemblance, thus indicating that results of rat growth assays may be applied to evaluate proteins in human

diets. It was later reported that differences in amino acid requirements of man and rat are relatively small (68). Thus, the growth of young rats provides a good indication of the value of protein foods fed to infants and children, since the amino acid requirements of this age group are more critical than those of adults (4).

Protein Efficiency Ratio:

Another very widely used method for evaluating dietary proteins is the protein efficiency ratio (PER) assay. The concept of PER was first introduced in 1919 by Osborne *et al.* (69), and the term was defined as grammes of body weight gain per gramme protein consumed. Originally, the method involved the determination of PER at different levels of protein intake, the maximum value being taken as the PER of the diet tested. Maximum values were reported to be attained at about 10 percent levels of protein intake (70). Campbell (4) recommends the use of this 10 percent level to obtain maximum PER values in *ad libitum* feeding trials.

Several factors affect the PER assay. These factors include age of rats, duration of assay period, sex of rats and protein levels in test diets. These factors were discussed in detail by Campbell (4) and recently by Vessal (66), and no attempt is made to present a discussion of them in the present review. It is important to mention, however, that these factors should be standardized, in

order to minimize differences in results due to differences in strains of rats used and laboratory conditions (4,5). Standardized methods were developed by Chapman _et al._ (71) and Derse (72). The conditions recommended for performing PER assays with a minimum of variation include: the use of a four-week assay period (71,73,74), the use of male rats of one strain (4,5,73) and the use of 10 percent protein (N x 6.25) level in diets adequate in other nutrients (4) and fed to the rats _ad libitum_ (4,5). The use of a casein standard has been also recommended (71,75). Rats between 20 and 23 days of age were recommended by Chapman _et al._ (71), while Derse (72) recommends 21 days old rats for PER determinations. Using these recommended conditions, it has been demonstrated that reproducible results can be obtained from experiments performed at different laboratories (4,76).

The following drawbacks of the PER method of determining protein quality were pointed out:-

a) Body weight gains may not represent constant composition for different proteins.

b) Results of PER may vary with dietary intake and protein level.

c) Full utilization of dietary protein is assumed.

d) The method does not permit the evaluation of dietary proteins that do not support growth. Campbell (4) points out, however, that the

determination is simple and fairly accurate, provided all experimental conditions are carefully standardized.

Net Protein Utilization:

This method of protein evaluation was first introduced in 1953 by Bender and Miller (77). It measures the amount of dietary protein incorporated in the body. The method consists of feeding 10 percent protein diets to weanling rats, ad libitum, for a ten-day period, after which nitrogen contents of the carcasses are determined. A control group, fed protein-free diet, is included simultaneously. NPU is defined as:-

$$\frac{(\text{Body N of test group}) - (\text{Body N of no-protein group}) + (\text{N consumed by non-protein group})}{(\text{N consumed by test group})}$$

A detailed description of the method was given by Miller and Bender (90). It was also reported that the ratio of nitrogen to water was constant in carcasses of rats from the same colony; thus another procedure was devised for the determination of NPU by estimating the water content of the carcasses (78).

The NPU method is subject to considerable variation, and many drawbacks were pointed out. Chapman (71) reported that NPU determinations are as variable as PER determinations. Campbell (4) points out that the method is insensitive to lysine deficiency. Miller and Naismith (57), however, argue that lysine deficiency will not affect the use of NPU in

evaluating the diets of developing countries, because of their observation that diets of such countries are usually more deficient in the sulphur-containing amino acids. Morrison _et al._ (79) have reported a direct relationship between NPU values and dietary lysine content; lower values being observed when vitamins and minerals were deleted from the test diets.

A high positive correlation between NPU and PER values was reported by Bender (80), who thus considers the two assays to give the same order of ranking proteins according to their nutritional quality.

MATERIALS AND METHODS

Test Diets

Laubina 103:

This is a protein-rich food mixture developed at the Division of Food Technology and Nutrition, American University of Beirut, for feeding infants and young children in the Middle East (81). This mixture consists of a basic mixture made of burghol[1] and chick-peas (Cicer arietinum), to which dry skim milk, vitamins A and D and minerals are added. Slight modifications were made on the original formula of this food mixture, to suit its use for animal assays. The composition of Laubina 103, both in its original and modified forms, is given in Table 1.

Laubina 103 was prepared by cooking burghol[2] and chick-peas[2] with water for one hour, in a double-jacketted steam kettle. The mixture was then dried at 95°C in a forced-draft oven for 24 hours, cooled and ground to a flour. Dry skim milk, corn oil, vitamins and minerals were then added and all were sufficiently mixed in a rotary mixer.

"B & L" :

This is a similar food mixture to Laubina 103, except that an equal quantity of lentils[2] (Lens esculenta)

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1. Local Arabic name for par-boiled wheat (Triticum vulgare).
2. Purchased from local market.

is used in place of chick-peas (cf. Table 1). The method of preparation was similar to that of Laubina 103.

Casein Diets:

Diets, in which the sole source of protein was casein, were also prepared. At all levels of dietary protein tested, these diets were isonitrogenous and isocaloric with other test diets.

For convenience, vitamins, minerals and corn oil were not usually mixed with other ingredients of Laubina 103 and "B & L", during the preparation of test diets. A basal diet of the following composition was prepared in all cases:-

Ingredient	Percentage
Corn starch	80
Corn oil	to make 5 or 10
Non-nutritive cellulose (Alphacel)[3]	5
Mineral mixture (USP XIV)[3]	4
"Vitamin Diet Fortification Mixture"[3]	1

In the preparation of test diets, the required quantities of Laubina 103, "B & L" (both without addition of vitamins, minerals and corn oil) or vitamin-free casein[4], to provide the protein level desired, were incorporated into the basal diet at the expense of corn starch. Crude

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3. Obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.

4. Obtained from General Biochemicals, Chagrin Falls, Ohio, U.S.A.

fat (ether extract) was adjusted to 10 percent in diets used for protein efficiency ratio (PER) and net protein utilization (NPU) assays, and to 5 percent in all other studies; by the addition of corn oil. Non-nutritive cellulose was added as required to make the different diets isocaloric.

Analytical Methods

Analysis of Diets:

Moisture, crude fat (ether extract), crude fibre and ash were determined in duplicates according to the methods described in the A.O.A.C.[5] Handbook, 1960. Nitrogen was determined by a modified macro-Kjeldahl method; crude protein being calculated by multiplying percent nitrogen in the sample by 6.25. The nitrogen-free extract (NFE) was calculated in the following manner:

NFE = 100 - (moisture % + crude fat % + crude protein % + crude fibre % + ash %).

Lysine, threonine and the sulphur-containing amino acids (methionine and cystine) were determined in Laubina 103 by the microbiological methods described by Block and Weiss (82). Only methionine and cystine were determined in "B & L" and casein.

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5. Association of Official Agricultural Chemists.

Carcass and Urine Analyses:

Nitrogen was determined in the carcasses of rats used for NPU assays by the same modified macro-Kjeldahl method used for diets. Liver and urine nitrogen was determined by a micro-Kjeldahl method (83). Urinary creatinine was determined colorimetrically by Gaff's reaction (colour formation with alkaline picrate) according to the method described by Kerr (84). Colour intensity measurements were then carried at 520 millimicra with a Bausch and Lomb Spectronic 20 colorimeter.

Urinary inorganic sulphur was determined by precipitation with benzidine and subsequent titration with standard sodium hydroxide, as described by Hawk, Oser and Summerson (85); with slight modifications.

Determination of Liver Xanthine Oxidase:

Liver xanthine oxidase (LXO) activity was determined at the beginning according to the method of Litwack _et al_. (86), based on the measurement of xanthine disappearance. This method was found inconvenient, however, and it was easier to measure LXO activity by determining uric acid formation. The latter was determined by the spectrophotometric method of Kalckar (87), based on the measurement of optical density change, at 293 millimicra, due to oxidation of uric acid by uricase.

The general plan of the assay, as described by Litwack _et al_. (86), was slightly modified and adopted in

both methods of determination. Differences between the two methods were in the deproteinizing agents used, and in the subsequent measurements on the protein-free filtrate obtained. An outline of LXO activity determination is given below:

The rats were slightly anaesthesized with ether and exanguined by cutting the major blood vessels of the neck. Livers were quickly excised in toto, gently pressed between filter papers to remove excess blood and then placed on cracked ice for several minutes. A portion was then blotted, weighed and homogenized in 5 volumes of ice-cold 0.05M sodium-potassium phosphate buffer, pH 7.4, for 1.5 minutes, in a VirTis[6] tissue homogenizer fitted with an ice-jacket. The homogenate was strained through a gauze and 5 ml were then added to each of the following mixtures:

Test mixture	Control mixture
0.6 ml 0.04M xanthine[7]	0.6 ml water
0.4 ml 0.05M phosphate buffer.	0.4 ml phosphate buffer.

After mixing, 2.5 ml aliquots were immediately taken from both test and control mixtures for the initial measurement. The remainder was incubated at 37°C for 2 hours (4 hours for low-activity livers) and another 2.5 ml

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6. VirTis Hi-Speed "45" homogenizer, Model 16 - 200. The VirTis Co., Inc., Gardiner, New York, U.S.A.

7. Obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. The free base or the sodium salt were used.

aliquot was taken from each mixture.

In the method of Litwack _et al_. (86), each aliquot was directly pipetted into a 25 ml volumetric flask containing 2.5 ml of 40 percent sodium tungstate and 15 ml of 0.33 N sulphuric acid. After mixing, the volume was brought to 25 ml with water and the contents were centrifuged. A 0.5 ml aliquot was then taken from the protein-free supernatant and added to a tube containing 2.5 ml of water and 1 ml of Folin-Ciocalteu reagent (diluted 1:1 with water before use (88)). Colour was developed by the addition of 5 ml of saturated sodium carbonate solution. The intensity of the blue colour produced was then measured against a reagent blank at 660 millimicra, with a Baush and Lomb Spectronic 20 colorimeter. Xanthine concentration was calculated from a standard curve, and the value obtained for the control was subtracted from that of test. LXO activity was calculated from the change in xanthine concentration over a period of one hour.

When LXO activity was calculated by determining uric acid formation, the aliquots taken from the reaction mixture were pipetted into centrifuge tubes containing equal volumes of 6 percent perchloric acid (87) and centrifuged. The protein-free supernatant was neutralized with sodium hydroxide and diluted 20 times with 0.07M glycine-sodium hydroxide buffer, pH 9.3 (10 times dilution for controls was enough). Uric acid was then determined as

follows (87): The optical density, E_1, of 3 ml sample was measured against a water blank, at 293 millimicra, with a Beckman DU spectrophotometer. Subsequently, 0.01 ml of a uricase[8] suspension in glycine-sodium hydroxide buffer (equivalent to 3-5 units of the enzyme) was mixed into the sample, and the mixture incubated at room temperature. After a period of time, sufficient to ensure complete oxidation of uric acid to allantoin, another measurement, E_2, was done. The extinction of the enzyme, E_3, was determined by adding 0.01 ml of the enzyme suspension to 2.99 ml of glycine-sodium hydroxide buffer and measuring the optical density against a buffer blank. The value for the enzyme extinction was always small, compared with that for the unknown. The decrease in optical density, E, due to the oxidation of uric acid is given by:

$$E = E_1 + E_3 - E_2$$

Under these conditions, the oxidation of one microgramme of uric acid will correspond to an optical density decrease of 0.075 (89). Values obtained for control were subtracted from those of test, and LXO activity was calculated from the amount of uric acid formed over one hour.

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8. Lyophilized preparation in ampoules, of 100 units each. Obtained from L. Light and Co., Ltd., Colnbrook, England.

Animal Experiments

The experimental animals used were weanling (21-23 days old), male, albino rats of the Sprague-Dawley strain[9]. After arrival by air, the animals were fed a stock diet[10] for a period of 1 - 2 days for recovery. In some experiments, when older rats were needed, they were fed stock diet for 10 - 12 days. In one experiment only, 80 - 85 days old rats were used, and were obtained at this age from the animal suppliers.

The animals were individually housed in mesh-bottomed cages in an air-conditioned laboratory, maintained at 70 ± 2° F and relative humidity of ca. 60 percent. In all experiments, animals were assigned to diets according to a randomized block design. Food and water were supplied ad libitum, and food intakes and body weight gains were recorded periodically.

In experiments involving urine collection, the animals were transferred to funnel-bottomed metabolic cages and urine was collected in glass beakers, each containing 2 ml of 2N hydrochloric acid, to prevent bacterial growth. Faeces and spilled diet were frequently removed to avoid contamination of urine. The bottoms of cages were washed with distilled water, at the end of every collection period, and the washings were added to urine collections.

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9. Obtained from Animal Suppliers (London), Ltd., London, England.

10. Obtained from Vitasni Feed Co., Beirut, Lebanon.

PER was determined by the standardized method of Chapman *et al*. (71). Corrected PER values were calculated as follows:

$$\frac{2.5}{\text{Determined PER of casein}} \times \text{PER (unknown)}$$

NPU determination was based on the method of Bender and Miller (78) and Miller and Bender (90). Groups of 8 animals each were assigned for each diet. A negative control group was fed a protein-free diet throughout the determination. After a 10 days period, the animals were sacrificed with chloroform; incisions were made in the cranium, thorax and abdomen of each animal, and the carcasses were dried in a forced-draft oven at 95-100^{0}C for 2-3 days. Each carcass was then separately ground in a meat-mincer twice or thrice to ensure homogeneity of mixture. Nitrogen was determined on a sample of the ground material. NPU was then calculated from the expression (78):

$$\text{NPU} = \frac{B - (B_k - I_k)}{I} \quad ;$$

Where B and B_k are the total body nitrogen of animals on test and protein-free diets, respectively, and I and I_k are their nitrogen intakes.

NPU (operative) was determined on rats kept on the diets as such, i.e., without any modification (*cf*. Table 1), and at their original protein levels.

Growth trials were carried out using 10 rats in

each group; at the original protein levels of Laubina 103 and "B & L".

The results of all experiments were analysed statistically. Significance of differences between means was tested by calculation of the least significant differences at 5 and 1% levels, for experiments involving control groups; and by Duncan's multiple range test, at 5 or 1% levels, for those experiments where all possible comparisons were made (91).

TABLES AND FIGURES

Table 1. Proportions of different ingredients in Laubina 103 and "B & L"[1].

Mixture	Bur-ghol	Chick-peas	Len-tils	DSM[2]	Corn oil	Vitamins[3] A & D	Vitamin mixture	Bone ash	Mineral mixture	Sucrose	Citric acid
	%	%	%	%	%	%	%	%	%	%	%
Laubina 103	60	20	___	10	5	1	___	1	___	2	1
Laubina 103[4]	60	20	___	10	5	___	1	___	4	___	___
"B & L"	60	___	20	10	5	1	___	1	___	2	1
"B & L"[4]	60	___	20	10	5	___	1	___	4	___	___

1. Values calculated on air-dry basis.
2. Dry skim milk.
3. 5000 International Units of vitamin A and 500 International Units of vitamin D in one gramme of sucrose.
4. Modified for the feeding of rats.

Table 2. Proximate composition of Laubina 103 and "B & L"[1].

Mixture	Moisture	Ether extract	Crude fibre	Crude protein	Ash	N.F.E.[2]
	%	%	%	%	%	%
Laubina 103	7.8	8.2	2.5	16.0	3.0	62.5
"B & L"	7.5	8.1	2.8	17.5	2.9	61.2

1. Values represent means of duplicate determinations, expressed on air-dry basis.

2. Nitrogen-free extract.

Table 3. Portion of rat requirement for three amino acids, contributed by Laubina 103 at 6 and 10 percent protein levels.

Amino Acid	Portion of requirement supplied by Laubina 103 at[1]:	
	6 percent protein level	10 percent protein level
	%	%
Lysine	56	90
Methionine + cystine	36	59
Threonine	32	56

1. Values were calculated by expressing the content of each amino acid per 100 grammes of Laubina 103, at 6 and 10 percent protein levels, as a percentage of the requirements of rat according to Rao _et al._ (93).

Table 4. Effects of amino acid supplementation on body weight gain of rats fed diets containing 10 percent protein from Laubina 103[1].

Group No.	Amino acid supplement			Food consumption[4]	Gain in weight[5]
	L-Lysine[2]	DL-Methionine	DL-Threonine		
	gm %	gm %	gm %	gm/rat	gm/rat
I[3]	———	———	———	179.2±17.4[6]	49.6±7.7
II	0.05	———	———	136.0±38.4	31.5±20.1
III	0.10	———	———	163.7±25.5	43.1±6.4
IV	0.20	———	———	162.7±21.5	42.0±11.1
V	———	0.10	———	166.2±23.6	37.5±7.9
VI	———	0.30	———	162.5±15.3	38.2±3.1
VII	———	0.50	———	161.5±24.8	36.7±11.8
VIII	———	———	0.10	155.8±36.4	33.0±15.8
IX	———	———	0.20	157.6±38.4	35.0±18.0
X	———	———	0.40	166.1±38.5	35.2±13.7
XI	0.10	0.30	0.20	156.0±18.7	33.2±9.4
XII	0.10	0.30	———	151.5±11.8	33.0±10.9
XIII	0.10	———	0.20	154.2±26.6	36.7±11.8
XIV	———	0.30	0.20	176.2±10.9	37.1±5.4

1. Six animals were assigned for each diet, in a 14-day experiment.
2. Added as L-lysine hydrochloride.
3. Control diet.
4. L.S.D. at 5% level = 28.4
5. L.S.D. at 5% level - 13.6
6. Standard deviation of the mean.

Table 5. Effects of amino acid supplementation on body weight gain of rats fed diets containing 6 percent protein from Laubina 103[1].

Group No.	Amino acid supplement			Food consumption[3]	Gain in weight[4]
	L-Lysine[2]	DL-Methionine	DL-Threonine		
	gm %	gm %	gm %	gm/rat	gm/rat
I	____	____	____	187.4±26.9[6]	6.2±7.9
II	0.20	____	____	203.4±27.5	6.0±9.8
III	0.40	____	____	188.6±23.1[7]	7.3±9.3
IV	0.60	____	____	230.6±34.7	8.8±4.9
V	____	0.20	____	219.9±39.1[7]	13.8±8.8
VI	____	0.40	____	203.9±29.1	10.7±10.2
VII	____	0.60	____	192.1±24.3	7.2±7.3
VIII	____	____	0.20	287.0±32.7[7]	22.0±1.4
IX	____	____	0.30	290.0±48.2[7]	33.0±1.7
X	____	____	0.40	263.9±24.5	39.2±7.4
XI	0.40	0.40	0.30	245.3±50.1[7]	48.7±3.6
XII	0.40	0.40	____	215.8±60.3[8]	16.8±4.0
XIII	0.40	____	0.30	251.6±61.5	41.3±3.0
XIV	____	0.40	0.30	267.9±26.5	44.2±1.3

1. Six animals were assigned for each diet, in a 28-day experiment.
2. Added as L-lysine hydrochloride.
3. L.S.D. at 1% level = 55.3 .
4. L.S.D. at 1% level = 10.3 .
5. Control diet.
6. Standard deviation of the mean.
7. Average of 5 animals.
8. Average of 4 animals.

Table 6. Protein efficiency ratio (PER), net protein utilization (NPU), operative net protein utilization (NPU_{op}) and growth values for rats fed diets containing protein from Laubina 103, "B & L" and casein.

Protein source	Protein level	PER[1] (corrected)	NPU[2]	NPU_{op}[2,3]	Growth[4,5]	
					Gain in weight	Food consumption
	gm %				gm/rat	gm/rat
Casein[6]	10.0	2.50	69.5±4.9[7]	———[8]		
Laubina 103	10.0	2.38±0.41	50.0±2.2			
	16.0			50.4±2.4	184.9±32.9	557.0±100.8
"B & L"	10.00	1.98±0.34	36.1±2.9			
	17.5			33.9±1.9	166.8±42.2	555.8±86.9
L.S.D. at 5% level		0.46			39.9	79.3
L.S.D. at 1% level			3.5	9.7		

1. 28-day assay period.
2. 10-day assay period.
3. Diets, without modification were used (cf. Table 1).
4. 42-day assay period.
5. Modified Laubina 103 and "B & L" were used (cf. Table 1).
6. Control diet.
7. Standard deviation of the mean.
8. Not determined.

Table 7. Effects of different diets on body and liver weights, liver nitrogen and xanthine oxidase activity of adult male rats[1].

Diet[2]	Nitrogen consumed	Gain in weight	Liver weight	Liver nitrogen	Xanthine oxidase activity[3]			
					Per gm liver	Total liver	Per gm liver nitrogen	Per 100 gm of rat
	gm/rat	gm/rat	gm	%				
Stock-diet[4]	____	____	12.31	2.71	3.00	36.90	111.11	13.60
Protein-free	____	-35.0	8.10	2.22	1.33	10.39	57.40	4.26
Casein	3.974	27.2	14.82	2.27	2.15	31.70	94.34	10.54
Laubina 103	3.892	23.8	12.20	2.57	2.34	29.19	93.26	10.09

1. Experimental period: 9 days. Values represent means of 5 animals/group.

2. All diets were isocaloric. Laubina 103 and casein contained 15.5 percent protein.

3. Expressed in micromoles of xanthine disappearance per hour.

4. Animals in this group were killed at the start of the experiment to obtain an idea about initial values.

Table 7a. Duncan's range test of the significance of differences between values given in Table 7.

Measurement	Comparisons	Significance level.
Gain in weight:	Protein-free Laubina 103 Casein	1%
Nitrogen consumption:	Laubina 103 Casein	5%
Liver weight:	Protein-free Laubina 103 Casein	5%
	Protein-free Laubina 103 Casein	1%
Liver nitrogen:	Protein-free Casein Laubina 103	1%
Liver xanthine oxidase:		
Per gm liver:	Protein-free Casein Laubina 103	5%
Per Total liver:	Protein-free Laubina 103 Casein	1%
Per 100 gm rat:	Protein-free Laubina 103 Casein	1%
Per gm liver N	Protein-free Laubina 103 Casein	1%

Table 8. Daily intake and excretion of nitrogen and sulphur in rats fed different diets[1].

Diet[2]	Body weight gm	Intake[3,4]				Excretion[3]					
		N	S_P	S_T	$1000\frac{S_T}{N}$	N	S	Cr[5].	$1000\frac{S}{N}$	$\frac{N}{Cr.}$	$\frac{S}{Cr.}$
Protein-free	254.4	—	—	2.5	—	39.8	1.1	2.9	27	14	0.38
Laubina 103	301.0	147	6.2	8.6	58	63.5	3.0	4.1	47	18	0.85
Casein	321.2	141	6.6	8.9	63	71.8	2.4	2.7	33	28	0.94

1. Experimental period: 9 days. Values represent means of 5 animals per group.

2. All diets were isocaloric. Laubina 103 and casein diets contained 15.5 percent protein.

3. Expressed in mg/100 gm rat/day. Values are means of three days period.

4. S_P represents the portion of sulphur in the diet derived from protein; S_T refers to the total intake of sulphur, derived from protein and from the mineral mixture (*cf.* Table 1).

5. Creatinine.

Table 8a. Duncan's range test of the significance of differences between the values given in table 8. Groups underlined by the same line do not differ significantly.

Measurement	Comparisons	Significance level
Body weight:	Protein-free Laubina 103 Casein	1%
Intake:		
N	Casein Laubina 103	5%
S_P	Laubina 103 Casein	5%
S_T	Protein-free Laubina 103 Casein	1%
Excretion:		
N	Protein-free Laubina 103 Casein	5%
	Protein-free Laubina 103 Casein	1%
S	Protein-free Casein Laubina 103	1%
Creatinine	Casein Protein-free Laubina 103	5%
1000 S/N	Protein-free Casein Laubina 103	1%
N/Creatinine	Protein-free Laubina 103 Casein	5%
	Protein-free Laubina 103 Casein	1%
S/Creatinine	Protein-free Laubina 103 Casein	5%

Table 9. Effect of different diets on body and liver weight, liver nitrogen and xanthine oxidase activity of male weanling rats[1].

Diet[2]	Body weight	Gain in weight	Nitrogen consumed	Liver weight	Liver nitrogen	Xanthine oxidase activity[3]			
						Per gm liver	Per total liver	Per 100 gm rat	Per gm liver nitrogen
	gm	gm/rat	gm/rat	gm	%				
Protein-free	33.2	-10.2	———	1.68	1.32	0.02	0.03	0.17	1.36
Laubina 103	70.7	29.2	1.664	3.23	2.35	1.17	3.78	5.35	49.80
Laubina 103 + 0.3% DL-threonine	70.0	25.8	1.717	3.27	2.64	1.32	4.32	6.17	50.04
"B & L"	89.2	35.0	2.357	3.93	2.52	1.06	4.17	4.68	42.10
"B & L" + 0.3% DL-methionine	91.7	37.7	2.341	4.22	2.48	1.56	6.58	7.16	62.87
Casein	85.7	31.0	1.880	4.28	2.78	1.20	5.20	6.02	43.70

1. These rats were used for NPU assay. Experimental period: 10 days. Initial body weight ranged between 40-55 gm. Values represent means of 6 animals per group.
2. All diets were isocaloric. Laubina 103, "B & L" and casein diets contained 10 percent protein.
3. Expressed in micromole uric acid formation/hour.

Table 9a. Duncan's range test of the significance of differences between values given in Table 9.

Measurement	Comparisons[1]	Significance level
Gain in weight:	PF LT L C BL BLM	1%
Nitrogen consumption:	L LT C BLM BL	5%
Liver weight:	PF L LT BL BLM C	5%
	PF L LT BL BLM C	1%
Liver Nitrogen	PF L BLM BL LT C	5%
	PF L BLM BL LT C	1%
Liver xanthine oxidase per gm liver	PF BL L C LT BLM	5%
per total liver	PF L BL LT C BLM	5%
	PF L BL LT C BLM	1%
per 100 gm rat	PF BL L C LT BLM	5%
	PF BL L C LT BLM	
per gm liver N	PF BL C L LT BLM	1%

1. PF stands for protein-free; L for Laubina 103; LT for Laubina 103 supplemented with 0.3% DL-threonine; C for Casein; BL for "B & L" and BLM for "B & L" supplemented with 0.3% DL-methionine.

Table 10. Effect of amino acid supplementation to Laubina 103, casein and "B & L" on body and liver weight, liver nitrogen and xanthine oxidase activity of male rats.[1]

Diet[2]	Body weight gm	Gain in weight	Nitrogen consumed	Liver weight	Liver nitrogen	Xanthine oxidase activity[3]			
						Per gm liver	Per total liver	Per 100 gm rat	Per gm nitrogen
	gm	gm/rat	gm/rat	gm	%				
Laubina 103	155.0	101.1	6.973	7.43	3.01	2.12	15.62	10.08	69.86
Laubina 103 + 0.3% DL-threonine	170.5	117.5	6.709	8.38	3.32	2.51	21.03	12.32	75.65
Laubina 103 + 0.3% DL-methionine	156.5	133.5	8.530	8.92	2.74	2.93	26.03	13.94	106.68
Laubina 103 + 0.3% L-Lysine[4]	195.3	142.8	8.605	7.92	2.56	1.88	14.87	7.61	73.25
"B & L"	150.6	95.6	7.053	6.72	3.71	2.04	13.70	9.14	55.02
"B & L" + 0.3% DL-methionine	145.0	91.0	6.682	6.30	2.60	3.19	19.96	13.74	121.71
Casein	144.5	91.3	5.467	7.12	3.53	2.58	18.29	12.86	72.87
Casein + 0.3% DL-methionine	205.0	150.6	6.682	10.68	3.72	3.00	32.04	15.63	80.71

1. These rats were used for PER assay. Experimental period: 28 days. Initial body weight ranged between 45-60 gm. Values represent means of 6 animals per group.
2. All diets were isocaloric and isonitrogenous. Protein level: 10 percent.
3. Expressed in micromole uric acid formation/hour.
4. Added as L-lysine hydrochloride.

Table 10a. Duncan's range test of the significance of differences between values given in Table 10.

Measurement	Comparisons[1]	Significance level
Gain in weight:	BLM C BL L LT LM LL CM	5%
Nitrogen consumption:	C CM BLM LT LM L BL LL	1%
Liver weight:	BLM BL C L LL LT LM CM	5%
Liver nitrogen:	LL BLM LM L LT C BL CM	5%
Liver xanthine oxidase:		
Per gm liver:	LL BL L LT C LM CM BLM	5%
Per total liver:	BL LL L C BLM LT LM CM	1%
Per 100 gm rat:	LL BL L LT C BLM LM CM	5%
Per gm liver N:	BL L C LL LT CM LM BLM	5%

1. L stands for Laubina 103; LL, Laubina 103 + 0.3% L-lysine; LT, Laubina 103 + 0.3% DL-threonine; LM, Laubina 103 + 0.3% DL-methionine; BL, "B & L"; BLM, "B & L" + 0.3% methionine; C, Casein and CM, Casein + 0.3% methionine.

Table 11. Effects of amino acid supplementation to diets containing 10 percent protein from Laubina 103, casein and "B & L" on PER and NPU values[1].

Diet	NPU[2]	PER[3]
Laubina 103	51.0	2.17
Laubina 103 + 0.3% DL-threonine	63.4	2.63
Laubina 103 + 0.3% DL-methionine	___[5]	2.40
Laubina 103 + 0.3% L-lysine[4]	___[5]	2.16
"B & L"	35.7	2.10
"B & L" + 0.3% DL-methionine	41.1	2.02
Casein	69.5	2.50
Casein + 0.3% DL-methionine	___[5]	3.38

1. Initial body weight ranged between 45-60 grammes. Values represent means of 6 rats per group.
2. Experimental period: 10 days.
3. Experimental period: 28 days.
4. Added as L-lysine hydrochloride.
5. Not determined.

Table 11a. Duncan's range test of the significance of differences between values given in Table 11.

Measurement	Comparisons[1]	Significance level
PER (corrected) :	BLM BL LL L LM C LT CM	5%
NPU :	BL BLM L LT C	1%

1. L stands for Laubina 103; LT, Laubina 103 + 0.3% DL-threonine; LM, Laubina 103 + 0.3% DL-methionine; LL, Laubina 103 + 0.3% L-lysine; BL, "B & L"; BLM, "B & L" + 0.3% DL-methionine; C, casein and CM, casein + 0.3% DL-methionine.

Table 12. Daily intake and excretion of nitrogen and sulphur in rats fed different diets supplemented with amino acids[1].

Diet[2]	Body weight gm	Intake[3,4]			Excretion[3]					
		N	S_P	S_T	N	S	Cr.[4]	$\frac{S}{N}$ X 1000	$\frac{N}{Cr.}$	$\frac{S}{Cr.}$
Protein-free	33.9	——	——	5.0	43.2	3.2	1.1	74	40	2.99
Laubina 103	66.3	249	10.6	17.0	45.5	4.0	1.5	88	30	2.67
Laubina 103 +0.3% DL-threonine	66.1	259	11.0	17.6	47.7	2.5	1.4	53	34	1.79
"B & L"	84.1	313	13.4	26.0	69.8	3.9	2.4	57	29	1.63
"B & L" + 0.3% DL-methionine	86.0	337	27.9	41.8	51.5	5.3	2.2	104	23	2.41
Casein	80.8	274	13.1	20.1	65.3	5.0	2.4	77	27	2.08

1. These rats were used for NPU assay. Values represent means of 6 animals per group.
2. All diets were isocaloric. A 10 percent protein level was used.
3. Expressed in mg/100 gm rat/day. Values are means of three days period.
4. S_P represents the portion of sulphur in the diet derived from protein; S_T refers to the total intake of sulphur, derived from protein and from the mineral mixture (*cf*. Table 1). Cr.: Creatinine.

Table 12a. Duncan's range test of significance of differences between the values given in Table 12. Groups underlined by the same line do not differ significantly.

Measurement	Comparisons	Significance level
Intake :		
N	L LT C BL BLM	1%
S_P	L LT C BL BLM	1%
S_T	PF L LT C BL BLM	1%
Excretion :		
N	PF L LT BLM C BL	5%
S	LT PF BL L C BLM	5%
Creatinine	PF LT L BLM BL C	5%
$\frac{S}{N}$ X 1000	LT BL PF C L BLM	5%
N/Creatinine	BLM C BL L LT PF	5%
S/Creatinine	BL LT C BLM L PF	1%

[1]. PF stands for protein-free; L: Laubina 103; LT: Laubina 103 + 0.3% DL-threonine; BL: "B & L"; BLM: "B & L" + 0.3% DL-methionine; C: casein.

Table 13. Daily nitrogen and sulphur excretion in rats fed 10 percent protein from different diets supplemented with amino acids[1].

Diet[2]	Body weight	Intake[3,4]			Excretion[3]					
		N	S_P	S_T	N	S	Cr.[4]	$\frac{S}{N}$ X 1000	$\frac{N}{Cr.}$	$\frac{S}{Cr.}$
Laubina 103	149.6	166	7.1	11.3	39.3	2.4	2.4	61	17	1.00
Laubina 103 + 0.3% DL-threonine	164.0	146	6.2	9.9	42.9	2.6	2.7	61	16	0.95
Laubina 103 + 0.3% DL-methionine	181.6	132	11.0	33.8	39.8	4.4	2.4	110	10	0.90
Laubina 103 + 0.3% L-lysine	183.7	145	6.2	9.9	38.7	2.7	2.4	70	16	1.10
"B & L"	145.5	170	7.4	14.3	39.2	2.2	2.6	56	15	0.84
"B & L" + 0.3% DL-methionine	140.1	173	14.5	47.1	35.6	3.3	3.6	92	10	0.91
Casein	139.6	137	6.5	10.0	49.0	2.5	2.3	51	21	1.09
Casein + 0.3% DL-methionine	197.0	122	10.7	31.7	33.3	3.4	2.3	103	15	1.50

1. These rats were used for PER assay. Values represent means of 6 animals per group.
2. All diets contained 10 percent protein.
3. Expressed in mg/100 gm rat/day. Values are means of three days period.
4. S_P: Portion of sulphur in the diet derived from protein; S_T: Total intake of sulphur derived from protein and from the mineral mixture (<u>cf</u>. Table 1).; Cr.: Creatinine.

Table 13a. Duncan's range test of the significance of differences between values given in Table 13.

Measurement	Comparisons[1]	Significance level
Body weight gain :	BLM C BL L LM LL LT CM	5%
Intake :		
N	CM LM C LL LT L BL BLM	5%
S_P	LL LT C L BL CM LM BLM	1%
S_T	LL LT C L BL CM LM BLM	5%
Excretion :		
N	CM BLM LL BL L LM LT C	5%
S	BL L C LT LL BLM CM LM	5%
Cr.	C CM LM L LL BL LT BLM	5%
$\frac{S}{N}$ X 1000	C BL L LT LL BLM CM LM	1%
$\frac{N}{Cr.}$	BLM CM BL LT LL L LM C	1%
$\frac{S}{Cr.}$	BL BLM LT L C LL CM LM	5%

1. L stands for Laubina 103; LT, Laubina 103 + 0.3% DL-threonine; LM, Laubina 103 + 0.3% DL-methionine; LL, Laubina 103 + 0.3% L-lysine; BL, "B & L"; BLM, "B & L" + 0.3% DL-methionine; C, casein and CM, casein + 0.3% DL-methionine.

Table 14. Effect of level of dietary protein on urinary excretion of sulphur[1].

Diet[2]	Body weight gm	Intake[3]		Excretion[3]					
		N	S	N	S	Cr.[4]	$\frac{S}{N}$ X 1000	$\frac{N}{Cr.}$	$\frac{S}{Cr.}$
5% casein[5]	90.7	80	3.9	45.2	0.3	2.0	6	23	0.14
10% casein	112.3	197	9.4	90.7	0.7	2.4	8	39	0.30
15% casein	122.2	283	13.5	112.8	1.0	2.0	9	56	0.51
5% Laubina 103	88.1	76	3.2	35.0	0.7	1.9	19	18	0.35
10% Laubina 103	108.0	210	8.9	67.5	1.5	3.2	23	21	0.48
15% Laubina 103	112.4	280	11.9	68.2	1.9	2.2	28	32	0.91

1. Experimental period: 10 days. Values represent means of 6 rats per group. Initial weights ranged between 94 - 97 grammes.
2. Inorganic sulphur (present mostly as magnesium sulphate) was omitted from the mineral mixture added to the diets. Equivalent Mg was added as Mg citrate.
3. Expressed in mg/100 gramme rat/day. Values represent means of three days.
4. Creatinine.
5. 5, 10 and 15% refer to level of dietary protein.

Table 14a. Duncan's range test of the significance of differences between values given in Table 14.

Measurement	Comparisons[1]	Significance level
Body weight gain :	L5 C5 L10 C10 L15 C15	5%
Intake :		
N	L5 C5 C10 L10 L15 C15	1%
S	L5 C5 L10 C10 L15 C15	1%
Excretion :		
N	L5 C5 L10 L15 C10 C15	5%
S	C5 L5 C10 C15 L10 L15	5%
Cr.	L5 C5 C15 L15 C10 L10	1%
$\frac{S}{N}$ X 1000	C5 C10 C15 L5 L10 L15	1%
$\frac{N}{Cr.}$	L5 L10 C5 L15 C10 C15	5%
$\frac{S}{Cr.}$	C5 C10 L5 L10 C15 L15	5%

1. C5, C10, and C15 refer to diets containing 5, 10 and 15 percent of protein from casein; L5, L10 and L15 refer to diets containing 5, 10 and 15 percent of protein from Laubina 103.

Table 15. Effect of amino acid supplementation on urinary excretion of sulphur[1].

Diet[2]	Body weight gm	Intake[3]			Excretion[3]					
		N	S	$\frac{S}{N}$ X 1000	N	S	Cr.[4]	$\frac{S}{N}$ X 1000	$\frac{N}{Cr.}$	$\frac{S}{Cr.}$
Casein	121.1	222	4.8	22	103	1.3	3.6	11	28	0.35
Casein + 0.15% DL-methionine	137.0	212	7.6	36	77	2.1	3.7	30	22	0.61
Casein + 0.3% DL-methionine	131.2	261	10.6	41	72	5.9	3.7	86	19	1.59
Laubina 103	114.2	290	5.4	19	79	1.9	3.2	24	27	0.64
Laubina 103 + 0.2% DL-methionine	118.0	260	8.2	31	92	3.8	4.0	41	23	0.95
Laubina 103 + 0.4% DL-methionine	117.0	290	12.7	44	93	6.5	3.9	70	24	1.65

1. Experimental period: 10 days. Initial body weight ranged between 92-98 grammes. Values are means of 6 animals per group.
2. All diets contained 10 percent protein.
3. Expressed in mg/100 gm rat/day. Values represent means of 3 days.
4. Creatinine.

Table 15a. Duncan's range test of the significance of differences between values given in Table 15.

Measurement	Comparisons[1]	Significance level
Body weight gain :	LMM LM C L CMM CM	5%
Intake :		
N	CM C LM CMM L LMM	5%
S	C L CM LM CMM LMM	5%
Excretion :		
N	CMM CM L LM LMM C	5%
S	C L CM LM CMM LMM	5%
Cr.	L C CM CMM LMM LM	5%
$\frac{S}{N}$ X 1000	C L CM LM LMM CMM	5%
$\frac{N}{Cr.}$	CMM CM LM L LMM C	5%
$\frac{S}{Cr.}$	C CM L LM CMM LMM	5%

1. C, CM and CMM stand for casein, casein + 0.15% DL-methionine and 0.30% DL-methionine respectively. L, LM and LMM stand for Laubina 103, Laubina 103 + 0.2% DL-methionine and 0.4% DL-methionine respectively.

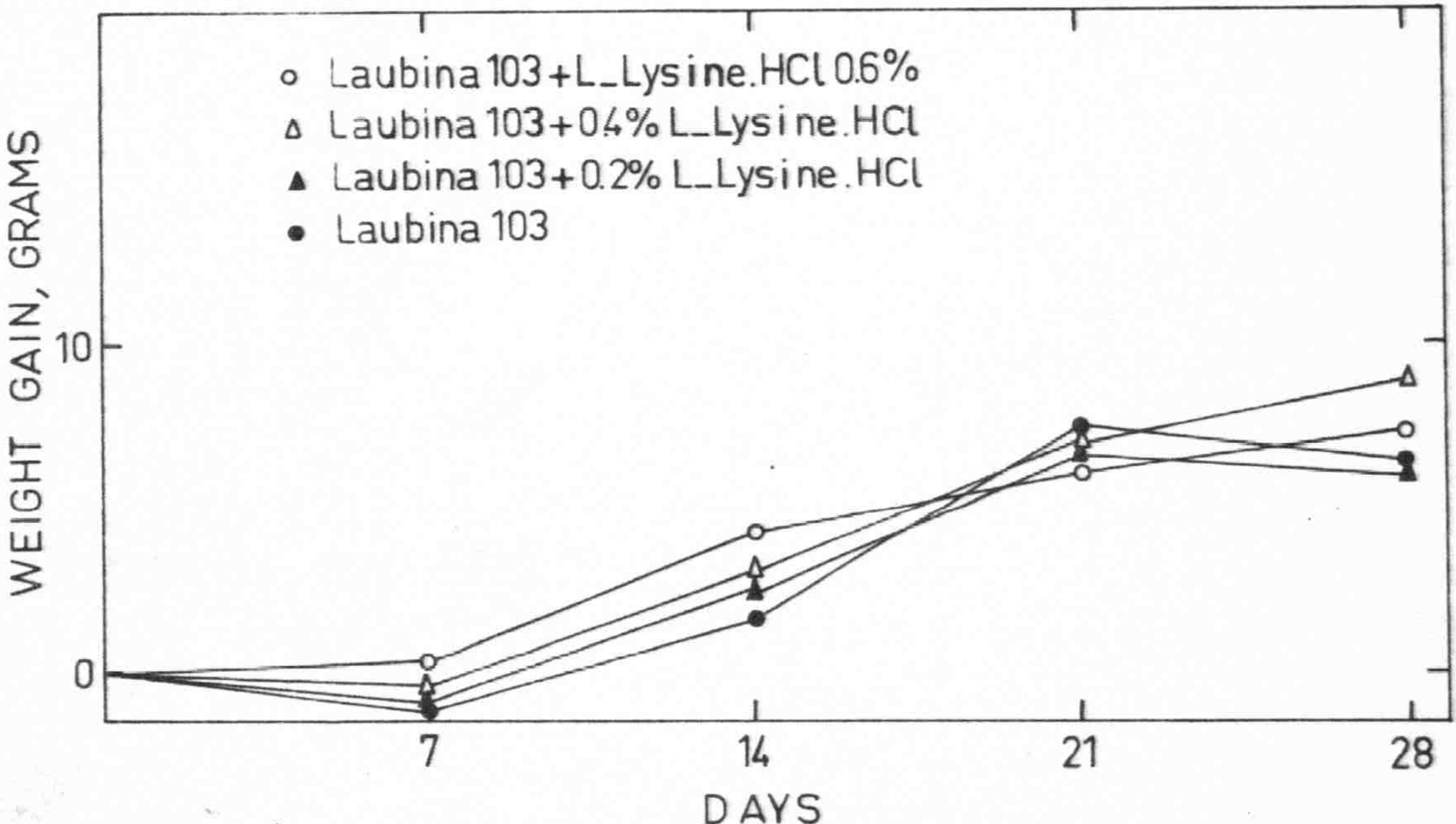

Figure 1. Growth curves of rats fed 6 percent protein from Laubina 103 supplemented with L-lysine.

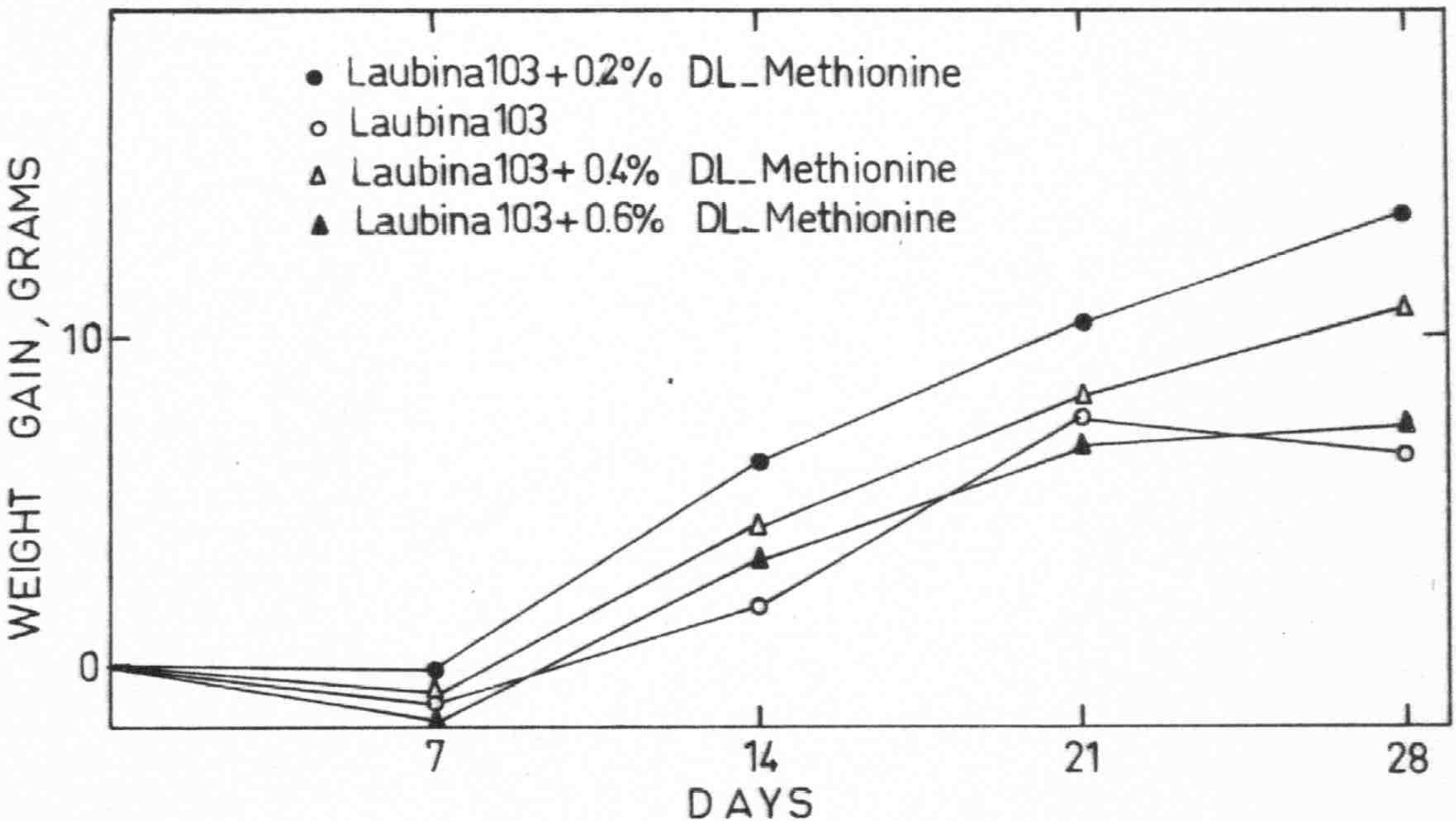

Figure 2. Growth curves of rats fed 6 percent protein from Laubina 103 supplemented with DL-methionine.

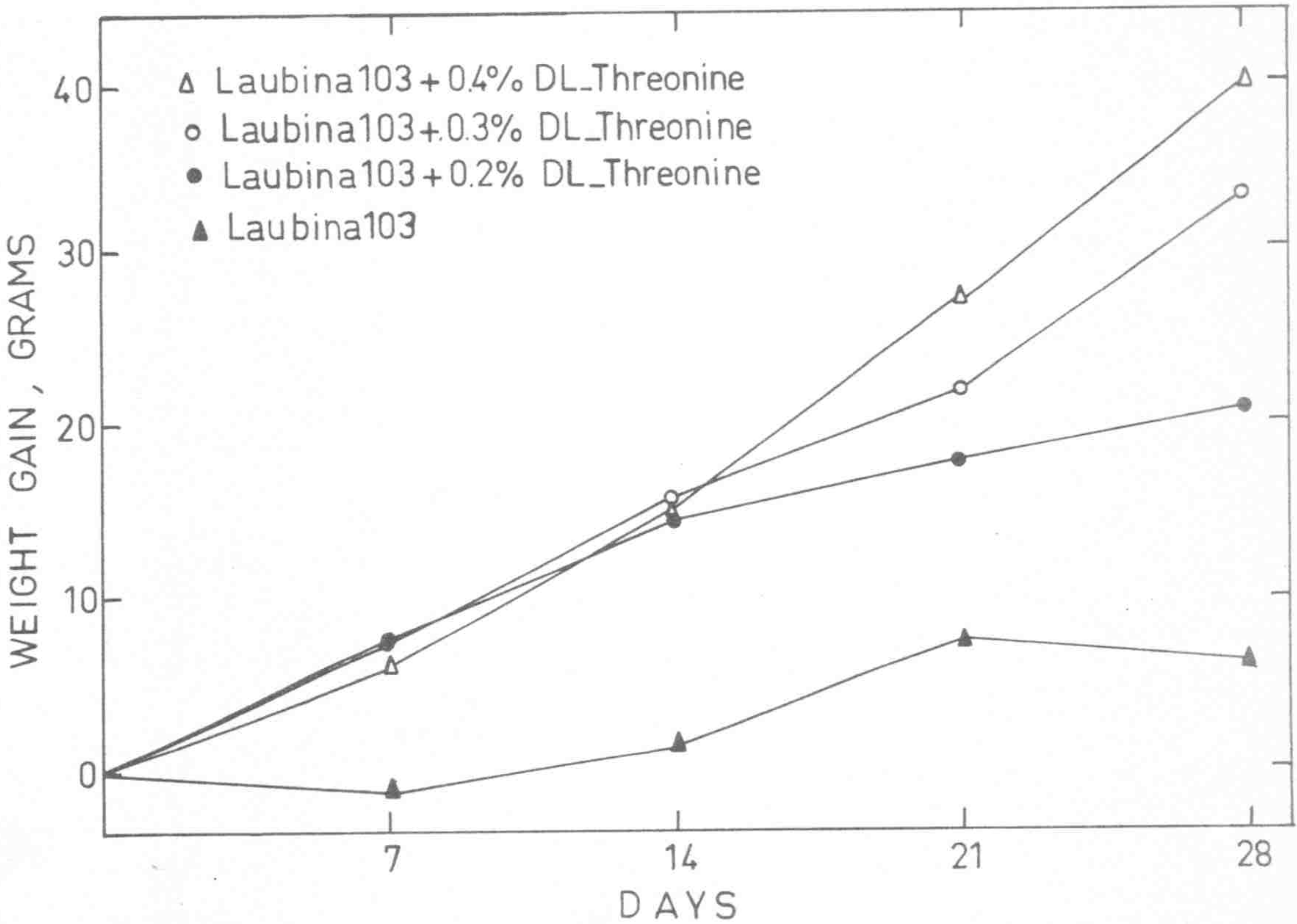

Figure 3. Growth curves of rats fed 6 percent protein from Laubina 103 supplemented with DL-threonine.

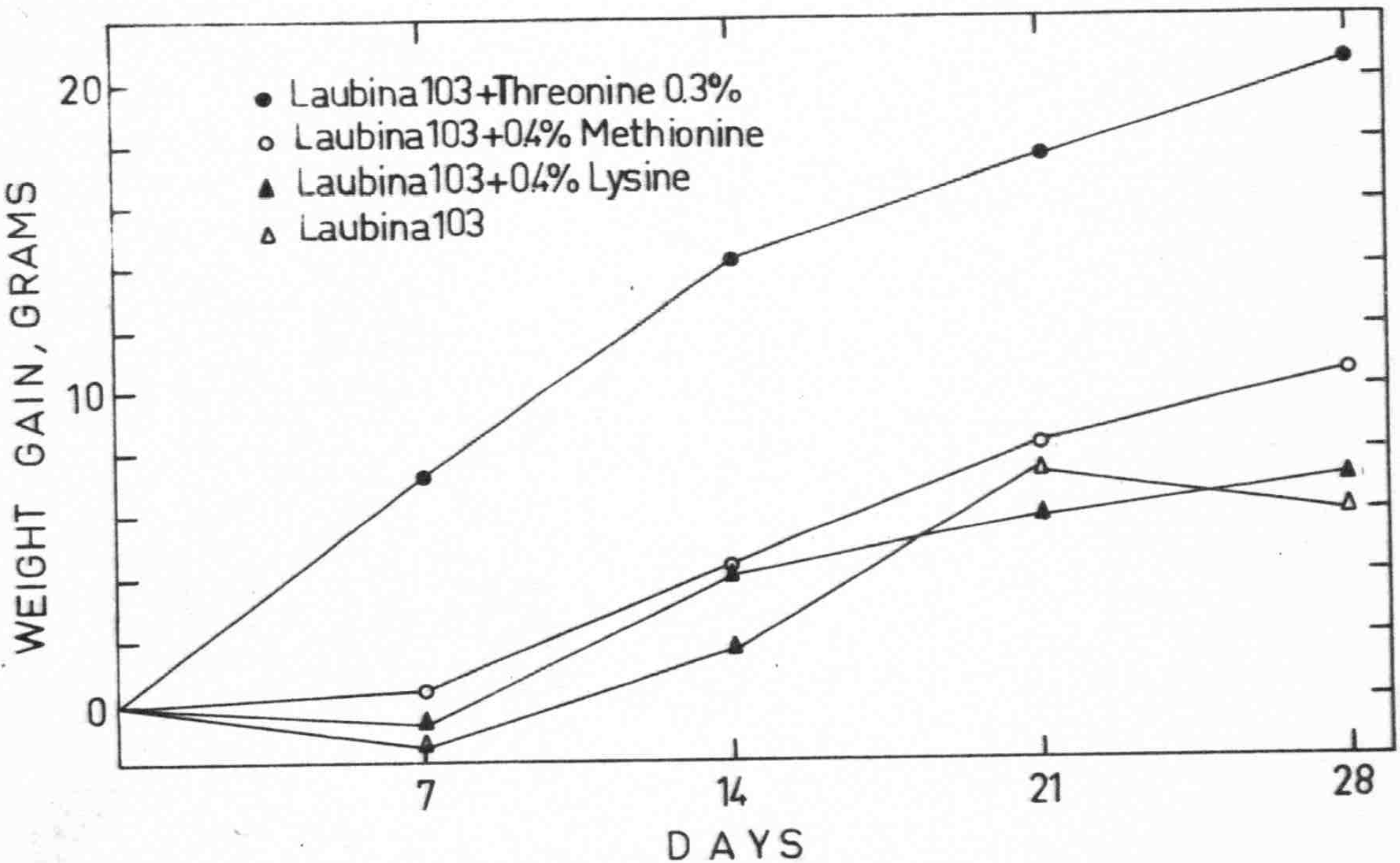

Figure 4. Growth curves of rats fed 6 percent protein from Laubina 103 supplemented with L-lysine, DL-methionine or DL-threonine.

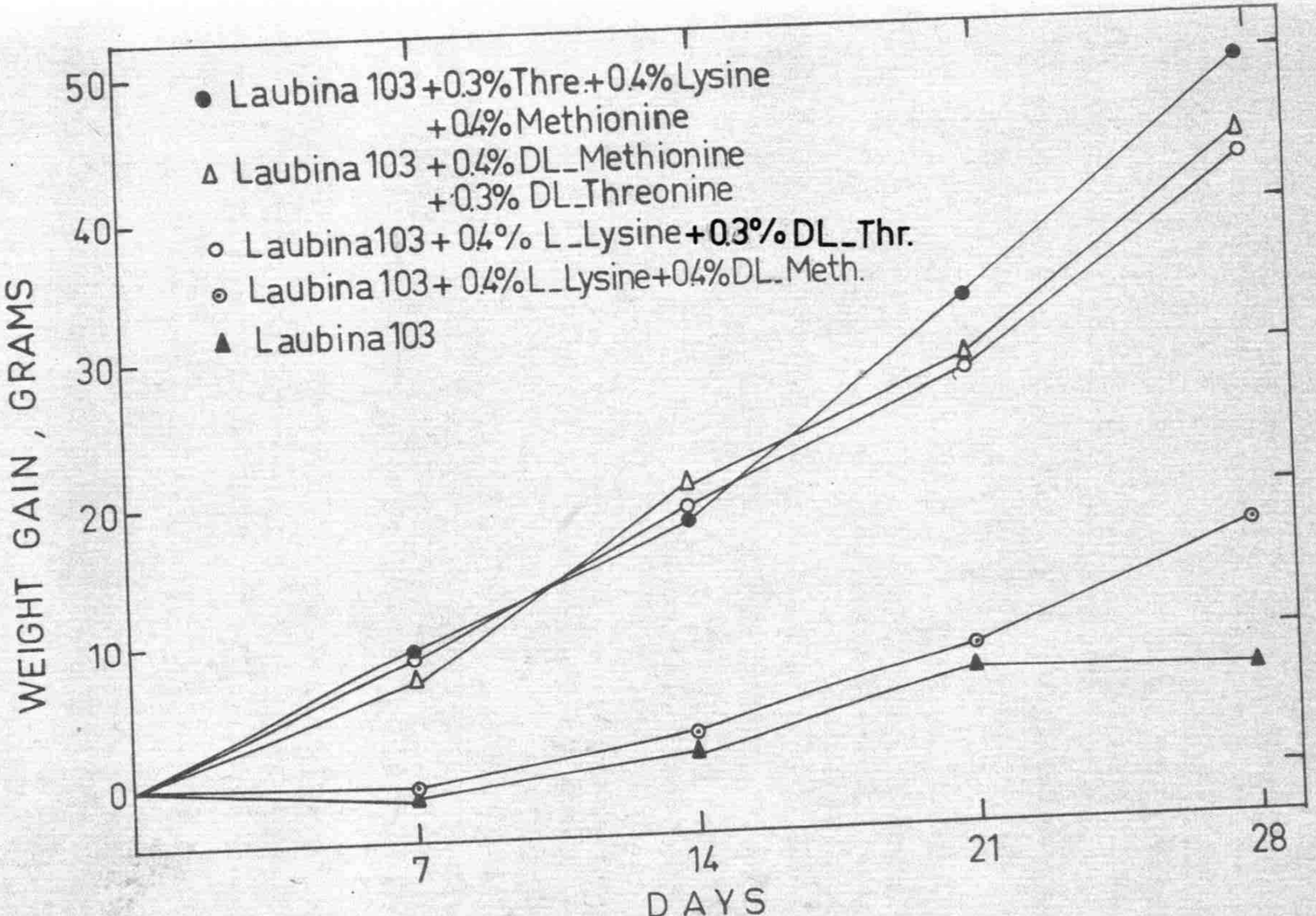

Figure 5. Growth curves of rats fed 6 percent ptotein from Laubina 103 supplemented with various combinations of amino acids.

RESULTS AND DISCUSSION

Determination of the Sequence in Which Amino Acids Become Limiting in Laubina 103:

Finding the limiting amino acids in the test diets used was needed at the start of this investigation. Casein was previously studied by Harper (92), who found that the sulphur-containing amino acids (methionine and cystine) in casein are the most limiting for the growth of rats, at low levels of protein intake (6 percent protein). At low protein levels, the effects of amino acid deficiencies in the diet become magnified.

From the amino acid composition of Laubina 103, threonine, the sulphur-containing amino acids and lysine, in this sequence, appeared to be the most deficient amino acids at the 6 and 10 percent protein levels (cf. Table 3), based on the requirements of rats according to Rao et al. (93). The sequence of the most and next limiting amino acids in the diet, predicted from the chemical composition, can be tested by measuring growth responses of animals fed low protein diets supplemented with amino acids individually, and then in combinations from which one amino acid at a time is omitted (92).

Two experiments were designed to study the sequence

in which threonine, the sulphur-containing amino acids and lysine, in Laubina 103, become limiting for the growth of the rat. Each of these amino acids was added to the basal diet at three levels, the middle level of which was calculated to raise the level of each amino acid to the rat requirement. Also, these middle levels were used when combinations of the three amino acids were added to the basal Laubina 103 diet.

When this experiment was carried out at 10 percent protein level in the diet, no significant differences in body weight gain were observed among the various groups after a period of two weeks. The results of this experiment are shown in Table 4. It was suspected that the lack of response of rats to the different diets may be due to the fact that the 10 percent protein level used was not low enough to exaggerate the deficiencies of the amino acids. Thus, in the next experiment, 6 percent protein level in the diet was employed, and rats were fed the diets for a period of four weeks. The results of this experiment are presented in Table 5, and the growth of the rats during the experiment is graphically represented in Figures 1-5.

Supplementation of lysine to the control diet (6 percent protein from Laubina 103) did not cause any significant increase in body weight of rats over that of the control group. Body weight gain due to lysine

supplementation increased as the level of lysine in the diet was raised (Figure 1), but the increase was insignificant statistically.

When methionine was added to the diet, there was a small, but insignificant, increase in body weight (L.S.D. at 5% level = 7.8), which decreased as the level of methionine in the diet was increased (Figure 2).

A highly significant increase in body weight, over that of the control group, was observed when threonine was added to the diet (L.S.D. at 1% level = 10.3). The growth of rats increased significantly as the level of threonine supplementation was increased from 0.2% to 0.3% or 0.4%, but insignificantly from the 0.3% to the 0.4% level. Figure 3 shows the growth of rats fed diets supplemented with different amounts of threonine. It could also be noticed that food consumption increased significantly when threonine was added to the diet (L.S.D. at 1% level = 55.3).

The highest gain in weight was observed when the rats were fed a diet supplemented with the three amino acids (Figure 5). When threonine was omitted from the combined supplement, a highly significant decrease in body weight gain was observed. Omission of methionine caused a slight insignificant decrease. The effect of the omission of lysine was less than that caused by the omission of methionine (cf. Figure 5).

The weight gain observed when methionine was added to the diet was not significantly higher (at the 5% level of probability) than that observed when lysine was added. However, the data presented suggest that the sulphur-containing amino acids are more limiting for the growth of rat than lysine, knowing that, at the 6 percent protein level, Laubina 103 is more deficient in the sulphur-containing amino acids (only 36% of requirement is available at this protein level) than in lysine, (provides 56% of the requirement), as determined by microbiological assays. Threonine is obviously the most limiting of the three amino acids, at this level of dietary protein.

Thus, the sequence in which amino acids in Laubina 103 become limiting for growth, as determined by microbiological assay of amino acids, is in agreement with results obtained by biological testing on rats.

Protein Efficiency Ratio and Net Protein Utilization:

To compare the results of the study of the effects of protein quality on liver xanthine oxidase (LXO) and urinary inorganic sulphur (UIS) excretion, several determinations were conducted on the same rats simultaneously.

Protein efficiency ratio and net protein utilization, two commonly used methods of protein evaluation, were employed in determining the protein quality of diets used in this study. The PER and NPU values for the

different diets fed are shown in Table 11, and the statistical significances of differences between them are given in Table 11a.

Values of PER for casein and Laubina 103 were not significantly different in this experiment, in agreement with the results of a previous determination (cf. Table 6). Casein had a higher PER than "B & L", but the value for Laubina 103 was not significantly higher than that of "B & L".

Supplementation of lysine to Laubina 103 did not increase its PER value, while a slight increase was observed when methionine or threonine was added. Methionine had no effect on the PER value of "B & L", but increased it significantly when added to casein.

The pattern of PER values was similar to that of NPU values. The highest values were obtained when casein was fed to the rats, followed by values obtained when Laubina 103 and "B & L" were fed, respectively. Supplementation of Laubina 103 with threonine, the most limiting amino acid, increased both the NPU and PER values, while supplementation of "B & L" with methionine increased only the NPU value, (cf. Table 11). Supplementing casein with methionine, the most limiting amino acid, increased its PER value.

These results indicate that casein has the highest protein quality, while "B & L" has the lowest. Also, it

could be observed that addition of the most limiting amino acids to a diet improves its protein quality.

Liver Xanthine Oxidase Activity:

Liver xanthine oxidase (LXO) activity was determined in adult rats in the first part of the study, and in the same rats used for PER and NPU assays later.

In the first experiment, adult (70 - 75 days old) male rats were fed a stock diet for a period of 7 - 10 days, then offered a protein-free diet, and 15.5 percent protein from each of Laubina 103 and casein for 9 days. Results of this experiment are shown in Tables 7 and 7a.

Rats fed the protein-free diet lost 35 grammes of their weight. Weight gains of rats fed casein were higher than those fed Laubina 103, but the difference was not significant. No significant difference was observed between the nitrogen consumption of the two groups.

The pattern of liver weights for the different groups was similar to that of body weights, being significantly higher in rats fed casein than in rats fed Laubina 103.

The percent nitrogen content of the group fed Laubina 103 was significantly higher than that of rats fed casein, but total liver nitrogen was not different.

The results shown in Table 7 show that no significant differences in LXO activity were observed between

rats fed Laubina 103 and those fed casein, when LXO activity was expressed in different terms. This is consistent with the fact that the two diets did not differ in their PER values.

Liver weight, nitrogen and LXO activity of the group fed protein-free diet were always lower than the other two groups (1% level of probability). However, the loss in LXO activity in this group was not as profound as reported by other workers (9, 53). The fact that LXO lost about 60% of its initial activity suggests that loss in activity is not only due to a general loss of liver protein, but also to the greater specific loss of enzyme protein (9, 11). This can be assumed from the fact that LXO activity per gramme of liver nitrogen was only about 30 percent of the initial value.

Litwack _et al_. (14) recommended the use of weanling rats in studies involving LXO, because it allows the study of the effect of protein in diets on liver protein while the animals are still in a generative state of tissue proteins. It was of interest to compare the effects of amino acid supplementation on LXO with the results obtained by other biological methods of protein evaluation, performed on the same animals.

In one experiment, weanling rats were fed diets containing 10 percent protein from Laubina 103, "B & L" and casein, with amino acid supplements added to "B & L" and

Laubina 103. Experimental conditions were chosen to allow NPU determinations on the same animals. The results of this experiment are shown in Tables 9 and 9a, and the NPU values are in Table 11.

Differences in body weight gains of the various groups of rats were insignificant at the 10th day of the experiment. Nitrogen intake for the group fed "B & L" was significantly higher than that of the groups fed Laubina 103 and Laubina 103 supplemented with threonine.

Addition of threonine to Laubina 103 increased the percent liver nitrogen significantly. Methionine addition, on the other hand, decreased the liver nitrogen of rats fed "B & L", but the difference was not significant. Liver nitrogen percent of the rats fed casein was significantly higher than those fed Laubina 103 and "B & L". The latter did not differ significantly between themselves, however.

LXO activity per gramme of liver did not differ among rats fed "B & L", Laubina 103 and casein (at the 5% levels of significance). The total LXO activity was significantly higher in the rats fed casein than in those rats fed Laubina 103 or "B & L". The activity of the enzyme per 100 grammes of rat and per gramme liver nitrogen did not differ in the three groups. Methionine was effective in raising LXO activity of rats fed "B & L". Threonine supplementation to Laubina 103 increased the activity per gramme of liver and per

100 gramme of rat, but not the total liver activity or the activity per gramme nitrogen. LXO activity of the protein-free group was always significantly lower than other groups (at 1% level of probability).

The data obtained from this experiment show that differences in the effects of casein, Laubina 103 and "B & L" on LXO were not significant, generally. The corresponding NPU values (cf. Table 11) were in the following descending order: casein, Laubina 103, "B & L", differences being highly significant.

In the next experiment, conditions were chosen to allow determinations of PER and LXO activity in the same rats. Laubina 103 was supplemented with 0.3% of each of lysine, threonine and methionine. Casein and "B & L" were each supplemented with 0.3% methionine.

The results of this experiment are shown in Tables 10 and 10a and the corresponding PER values in Tables 11 and 11a. The pattern of weight gains of rats fed Laubina 103 to which were added lysine, methionine and threonine was different from that expected from inspection of Table 5. This may be due to differences in nitrogen intake, however. The highest weight gain was observed in rats fed casein supplemented with methionine.

Liver weights of rats fed Laubina 103 were higher than those fed "B & L". The casein group showed an

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insignificant increase in liver weight over the group fed "B & L". Threonine and methionine significantly increased liver weights of rats fed Laubina 103, but the effects of the two amino acids were not different at the 5% level of probability. Lysine produced an insignificant increase in liver weight in rats fed Laubina 103. Methionine decreased liver weight when added to "B & L", but the effect was insignificant.

The pattern of response of liver nitrogen percent to the different diets was in the following descending order: "B & L, casein, Laubina 103; which is the opposite of the order in which liver weights could be arranged. Addition of methionine to "B & L" and Laubina 103 decreased liver nitrogen significantly. Lysine addition to Laubina 103 significantly decreased liver nitrogen, while threonine caused an increase.

LXO activity per gramme of liver was higher in the casein fed rats than in those fed Laubina 103 and "B & L", but the latter two groups were not significantly different from each other. Lysine decreased LXO activity per gramme of liver, when added to Laubina 103, while threonine increased the activity significantly. The addition of methionine produced significant elevation in LXO activity when added to any of the diets studied. The total LXO activity, and activity per 100 grammes of rat did not differ significantly for rats fed "B & L", Laubina 103 and casein.

The effects of amino acid supplementation on total LXO activity were similar to their effects on LXO activity, expressed per gramme liver. These effects, however, were generally less pronounced when the activity was expressed per 100 grammes of rat.

The results show that LXO activity per gramme liver nitrogen was significantly lower in the "B & L" group than the Laubina 103 and casein groups. The latter two groups did not differ significantly from each other, however. The LXO activity per gramme of liver nitrogen was significantly increased when threonine was added to Laubina 103. The same was observed when methionine was added to all diets tested.

Observations obtained from the two experiments described above indicate that methionine addition was effective in increasing the PER value and LXO activity of casein. That this effect is specific for LXO is shown by the works of Liener et al. (48), and others (49-51). Tamura et al. (79) reported significant increase in LXO activity in rats fed 9 percent protein diets when threonine or methionine, was added, but a decrease when lysine was supplemented. This same effect was observed in the present investigation. Lysine decreased LXO in rats fed Laubina 103 only when the enzyme activity was expressed per gramme liver and per 100 grammes of rat. It seems that the effect of lysine on LXO is through its effect in decreasing total

liver nitrogen (protein) rather than a specific effect on the enzyme. This is suggested by the fact that total liver nitrogen of animals fed Laubina 103 was higher than that of the lysine supplemented group. Furthermore, LXO activity per gramme of liver nitrogen was slightly increased when lysine was added to the diet.

Threonine increased LXO activity in rats fed Laubina 103 probably due to the fact that, being the most limiting amino acid in this diet, it would raise its nutritive value enough to affect the over-all anabolism of tissue proteins, including xanthine oxidase. This does not exclude the possibility that threonine may have a specific influence on LXO, however.

Addition of methionine to casein, Laubina 103 and "B & L" caused an increase in LXO activity. An increase in body weight gain and PER values of rats fed casein supplemented with methionine was observed. Similar increases were not observed in rats fed "B & L". This difference suggests that methionine (the sulphur amino acids) may not be limiting in "B & L". The presently reported observations establish the fact that the sulphur-containing are limiting in casein (92). Also, it indicates that the addition of methionine specifically increases LXO activity, regardless of the type of protein present in the diet.

Urinary Inorganic Sulphur:

Urines of the same rats used for LXO determinations were collected three days before the animals were sacrificed and were analyzed for nitrogen, creatinine and inorganic sulphur.

The results of the experiment made on adult rats are given in Tables 8 and 8a. It can be observed that nitrogen, and sulphur intakes were not significantly different in rats fed Laubina 103 or casein. Sulphur was present in the protein-free diet only in the form of inorganic sulphate, present in the mineral supplement.

Nitrogen excretion was significantly decreased when the rats were fed a protein-free diet. This nitrogen represents the nitrogen derived from the catabolic processes of tissue protein, enhanced by the absence of protein in the diet (1). Rats fed Laubina 103 or casein showed no significant differences in their daily nitrogen excretion.

Similar to nitrogen, inorganic sulphur excretion was not different for the rats fed Laubina 103 or casein, but was significantly lower for those fed the protein-free diet (at 1% level).

Creatinine excretion was significantly higher in rats fed Laubina 103 than in the other two groups. The ratio of nitrogen to creatinine in the urine of rats fed casein was higher than that of rats fed Laubina 103 or the protein-free diet. This is mainly due to the difference

in creatinine excretion between the two groups, rather than a difference in nitrogen excretion. The ratio was not significantly higher (at 1% level) for rats fed casein. This result may explain why the nitrogen-to-creatinine ratio of rats fed Laubina 103 was not significantly higher than that of rats fed the protein-free diet.

The ratio of $\frac{S}{N}$ X 1000 in the urines of the three groups were in the following ascending order: protein-free, casein, Laubina 103; and all the three groups were significantly different from each other (at 1% level). The difference between the casein fed rats, and rats which were fed Laubina 103 can be explained by the fact that the sulphur-containing amino acids are the most limiting amino acids in casein. Metabolic wastage of amino acid sulphur is expected to be greater when dietary protein is not deficient mostly in the sulphur-containing amino acids. Sulphur-containing amino acids were shown in the early part of this investigation to be the second-limiting amino in Laubina 103, next to threonine.

The ratio of inorganic sulphur to creatinine in the urines of rats fed casein was higher than that of the rats fed Laubina 103, but the difference was insignificant. Assuming that this ratio may reflect both the quantity and quality of dietary proteins, it could be noticed then, that these results are in agreement with the PER values, where the PER value of casein was found higher than that

of Laubina 103, but insignificantly (cf. Table 6). The results also agree, in general, with the LXO activity of the same rats (cf. Tables 7 and 7a).

The next experiment was carried out with rats on which NPU and LXO assays were made.

The results of this experiment are shown in Tables 12 and 12a. Nitrogen intakes of rats fed "B & L" and "B & L" supplemented with methionine was not significantly different. These two groups, however, consumed significantly (at 1% level) higher quantity of nitrogen than rats fed casein, Laubina 103 or Laubina 103 supplemented with threonine. Nitrogen consumption of the latter three groups did not differ significantly.

Total sulphur intakes of rats fed "B & L" and "B & L" supplemented with methionine were significantly higher than rats fed other diets. The only source of sulphur in the protein-free diet was the inorganic sulphur derived from the mineral mixture.

Nitrogen excretion of rats fed casein and "B & L" did not differ significantly but was higher than that of rats fed Laubina 103 and protein-free diets. Threonine addition had no effect on nitrogen excretion. Addition of methionine significantly decreased nitrogen excretion of rats fed "B & L".

Inorganic sulphur excretion was significantly higher in rats fed casein than when Laubina 103 or "B & L"

were fed, but no significant difference was found between the excretion of inorganic sulphur in rats fed a protein-free diet and that of "B & L" and Laubina 103 groups. Threonine supplementation to Laubina 103 decreased inorganic sulphur excretion significantly. As it would be expected, methionine increased inorganic sulphur excretion when added to "B & L".

Creatinine excretion was significantly higher in rats fed casein and "B & L" than in those fed Laubina 103.

The ratio of nitrogen to creatinine in rats fed protein-free diet had the highest value, and all other values did not differ significantly.

Casein and Laubina 103 did not differ in their effect on urinary $\frac{S}{N}$ X 1000 ratios, but both had significantly higher values than the "B & L" group. Opposite to its effect on the nitrogen-to-creatinine ratio, threonine decreased the ratio of sulphur to nitrogen significantly. This may be due to better utilization of the sulphur-containing amino acids in general when the limiting amino acid is added. On the other hand, addition of methionine to "B & L" increased the $\frac{S}{N}$ X 1000 ratio. This is expected, since the addition of methionine increases the dietary intake of sulphur.

Inorganic sulphur-to-creatinine ratio was higher for animals fed Laubina 103 than for the casein fed group. "B & L" was not significantly different from casein.

Again, threonine decreased the ratio of inorganic sulphur in urines of rats fed Laubina 103. Since creatinine excretion was not significantly different in these two groups, it becomes apparent that the difference is due to lower sulphur excretion (cf. Tables 12 and 12a). Rats fed casein and "B & L" did not differ significantly in their sulphur-to-creatinine values. Threonine supplementation to Laubina 103 resulted in a significant decrease, while methionine, on the other hand, has increased the value for "B & L". The highest ratio was observed for the protein-free group.

Next, it was of interest to extend the study to rats used for the PER and LXO studies. The results of this experiment are shown in Tables 13 and 13a.

Differences in body weight gains of the different groups were insignificant; except for the rats fed casein supplemented with methionine, which gained significantly more than any other group.

Sulphur intake of rats fed diets supplemented with methionine was significantly higher than all other groups.

Nitrogen excretion did not differ significantly among the various groups; except in the rats fed casein, where it was higher than other groups.

Inorganic sulphur excretion did not differ in rats fed casein, Laubina 103 or "B & L". Also it was observed that lysine and threonine had no effect when added to

Laubina 103. However, inorganic sulphur excretion was increased significantly when methionine was added.

Creatinine excretion was higher in rats fed "B & L" (significant at the 5% level), but did not differ significantly between all other groups. The ratios of inorganic sulphur/N in the urines of rats fed casein, Laubina 103 or "B & L" were not significantly different. However, the ratio was increased significantly when methionine was added to the diets. Lysine and threonine did not increase this ratio in rats fed Laubina 103.

Rats fed casein had higher nitrogen-to-creatinine ratio than all other groups. However, rats fed Laubina 103 or "B & L" showed no significant differences; but the ratio was significantly decreased when methionine was added to "B & L".

When casein was fed, the rats showed a higher inorganic sulphur-to-creatinine ratio than rats fed Laubina 103 or "B & L". This ratio was always increased when methionine was added to the diet. The increase was not significant, however, when methionine was added to the diets of rats fed "B & L". Threonine had no effect on the ratio of inorganic sulphur to nitrogen, while lysine caused an increase.

The results of this experiment indicate that only the addition of methionine to the diet causes an increase in the excretion of sulphur, and sulphur-to-nitrogen and

sulphur-to-creatinine ratios, except in the case of Laubina 103. Thus the excretion of sulphur in the urine is affected mainly by the dietary intake of sulphur (methionine).

It was then intended to omit inorganic sulphur, provided by the mineral mixture, from the diet; and to study the effects of different levels of dietary sulphur and protein on urinary sulphur excretion. It was calculated that 99 percent of the inorganic sulphur in the mineral mixture was provided by magnesium sulphate. Thus, magnesium sulphate was substituted by magnesium citrate, to provide almost the same amount of magnesium.

Results of the effect of dietary protein level on urinary inorganic sulphur excretion are shown in Table 14.

Three levels of dietary protein; namely, 5, 10 and 15 percent were provided by Laubina 103 or casein. The diets were fed for a period of 10 days, and urine was collected during the last three days of the experiment.

Since sulphur in the diet is derived only from dietary protein, the pattern of sulphur intake was similar to that of nitrogen, namely, increasing with increase in dietary protein.

Nitrogen excretion in the urine increased significantly as protein level in the diet was increased. The same pattern was observed for the ratio of $\frac{S}{N}$ X 1000 in the urine. The ratio of $\frac{S}{N}$ X 1000 was not significantly different

for rats fed 5 and 10 percent protein from casein, but both were lower than that of rats fed 15 percent protein from casein. However, the ratio for the three protein levels was significantly different in the three groups of rats fed Laubina 103, and were significantly higher than those fed casein.

The ratio of nitrogen to creatinine and the ratio of sulphur to creatinine increased significantly as protein level in the diet was increased. At the same level of protein, the ratio of sulphur-to-creatinine for Laubina 103 was significantly higher.

These results indicate that as the level of protein in the diet is increased, and thus the levels of sulphur and nitrogen, also, there is an increase in the excretion of nitrogen, sulphur, the ratio of $\frac{S}{N}$ X 1000, nitrogen-to-creatinine and sulphur-to-creatinine.

Thus, it is clear from these results that sulphur-to-nitrogen and sulphur-to-creatinine ratios are not an indication of the quality of the protein, but are a function of the dietary protein intake.

Results of the effect of methionine supplementation on urinary sulphur excretion are shown in Tables 15 and 15a. Two diets, casein and Laubina 103 were supplemented with DL-methionine. Three levels of supplementation were used with each diet, the middle level of which was calculated to meet the requirements of the rat for sulphur-containing

amino acids. Then two other levels, one lower and one higher, were used for comparison.

Dietary sulphur and sulphur-to-nitrogen ratio increased as the amount of methionine was increased in the diets.

A similar pattern was observed for the excretion of sulphir, sulphur-to-nitrogen and sulphur-to-creatinine ratios; namely an increase with the increase of methionine added to the diet. Similar patterns were observed for the two diets employed. Again these results indicate that the excretion of sulphur, sulphur-to-nitrogen and sulphur-to-creatinine ratios vary with the dietary intake of sulphur amino acids.

A comparison of the results obtained from this investigation is given in the table shown next page.

The $\frac{S}{N}$ X 1000 ratio may be taken as a measure of dietary protein quality only in some cases, because when sulphur amino acids are deficient in a diet, the $\frac{S}{N}$ X 1000 ratio will decrease. It is also apparent that the ratio decreases as the rats get older. This trend may be explained by the fact that younger rats are in a generative state of tissue protein and, thus, will be in positive nitrogen balance. Improved nitrogen retention, due to addition of the most limiting amino acid in the

Table 16. Comparison of the results obtained by different studies.

Diet	Rats used for NPU			Rats used for PER			Adult rats	
	NPU	LXO/ gm N	$\frac{S}{N}$ X 1000	PER	LXO/ gm N	$\frac{S}{N}$ X 1000	LXO/ gm N	$\frac{S}{N}$ X 1000
Laubina 103	51	50	88	2.17	70	61	93	47
Laubina 103 + 0.3% DL-threonine	63	50	64	2.63	76	61	—	—
Laubina 103 + 0.3% DL-methionine	—	—	—	2.40	107	110	—	—
Laubina 103 + 0.3% L-lysine	—	—	—	2.16	73	70	—	—
"B & L"	36	42	58	2.10	55	56	—	—
"B & L" + 0.3% DL-methionine	41	63	104	2.02	122	93	—	—
Casein	69	44	79	2.50	73	51	94	33
Casein + 0.3% DL-methionine	—	—	—	3.38	81	103	—	—

diet, was observed when threonine was added to Laubina 103 and when methionine was added to casein especially in rats used for NPU assay.

The $\frac{S}{N}$ X 1000 ratio was increased when methionine was added to the diets. This increase however, is mainly due to increased intake of dietary sulphur and to slight decrease in nitrogen excretion generally.

Changes in liver xanthine oxidase activity per gramme of liver seem to be the most suitable way of expressing the activity of the enzyme, since it reflects changes in the enzyme protein relative to liver protein content. Expressed as per gramme nitrogen, LXO activity increased with the age of the animals, as was observed by Westerfeld et al. (55). The LXO activity expressed in this manner in rats used for PER determination followed generally the pattern of $\frac{S}{N}$ X 1000 ratio in the urine.

The use of LXO activity and urinary $\frac{S}{N}$ X 1000 ratio in evaluating quality of protein in the diets was not as effective as PER or NPU. Both methods, namely, LXO activity and urinary $\frac{S}{N}$ X 1000 ratio were specifically affected by methionine addition to the diet, but did not reflect the effects of adding other amino acids to the diet.

The use of PER and NPU seem to be more effective in ranking diets according to their protein quality. The ease of performing these two assays, compared with the more elaborate LXO determination and urine collection in rats,

suggests the common use of PER and NPU methods. Differences in protein quality seem to compare more favorably with PER and NPU values. NPU values, however, showed more significant differences than PER values.

SUMMARY AND CONCLUSIONS

The limiting amino acids for the growth of rat in Laubina 103 were found to be threonine, methionine and lysine, successively.

Protein efficiency ratio (PER) values for Laubina 103 and "B & L" were 2.17 and 2.10, respectively; and the net protein utilization (NPU) values were 51.0 for Laubina 103 and 35.7 for "B & L". Addition of threonine to Laubina 103 increased the PER value to 2.63, while addition of methionine increased PER value of casein to 3.38 .

Liver xanthine oxidase (LXO) activity per gramme of liver nitrogen of rats used for PER assay was increased significantly by the addition of methionine to the diets. Values observed for this increase were 70 to 107, for Laubina 103; 55 to 122, for "B & L" and 73 to 81 for casein. However, a severe loss in LXO activity was observed when a protein-free diet was fed to the rats. The activity of LXO was also observed to increase with age.

Similar to LXO activity, the ratios of urinary inorganic $\frac{S}{N}$ X 1000 in rats used for PER assay were increased significantly by the addition of methionine to the diets. The increases in values were as follows: from 61 to 110, for Laubina 103; 56 to 93, for "B & L", and 51 to 103 for

casein. Thus the pattern of values for urinary inorganic $\frac{S}{N}$ X 1000 was similar to that of LXO activity values, in rats used for PER assay.

It was concluded that PER and NPU assays provide satisfactory methods of ranking diets according to their protein quality. LXO activity and the ratio of inorganic sulphur-to-nitrogen in the urine were useful in studying the effects of methionine supplementation to the diets on the quality of dietary protein.

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