EVALUATION OF LEAF PROTEIN USING THE ORGANISM *TETRAHYMENA PYRIFORMIS W*

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ABSTRACT

A protozoal method for the evaluation of protein quality was tested using the organism Tetrahymena pyriformis W. Mechanical extraction of leaves of the plant Chenopodium, produced a protein concentrate in the form of a press-cake. Portions were either refrigerated, frozen, freeze-dried, decolorized with acetone or oven-dried.

The criterion used to measure protein quality was a direct microscopic count of organisms as compared to that for an isonitrogenous amount of vitamin-free casein.

Aminograms of the concentrates compared favorably with vitamin-free casein. Essential amino acid content appeared to decrease slightly with processing. DL-methionine, the limiting amino acid, was added to all treatments as were L-isoleucine and L-lysine. All supplements were added singly and in all combinations.

Unsupplemented leaf proteins supported poor growth except for the decolorized sample. It is hypothesized that the presence of free fatty acids or chlorophyll breakdown products in the green leaf preparations may have been inhibitory.

Analysis of variance of supplementation data showed improved growth for all treatments on addition of methionine and lysine, and little effect for addition of isoleucine. The methionine-lysine combination generally resulted in significantly improved growth but the supplemented decolorized samples provided the best growth of all leaf samples tested.

Results of this study demonstrate that leaf protein could supplement low quality diets in a significant way and that Tetrahymena pyriformis, has the ability to reflect protein quality with reasonable accuracy.

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INTRODUCTION

The shortage of protein in human diets is the most crucial aspect of the present world food problem. Over half the world's population subsists on cereal diets that are deficient in protein. To meet the challenge of supplying low cost high quality protein foods for the protein-hungry people of the world, it is necessary to apply ourselves vigorously to seek out means whereby we can combine nutritional knowledge with technological advances. One approach is to bypass the animal and direct our attention to the mechanical production of plant protein concentrates of maximal protein quality.

Mechanical extraction of protein from various types of leaves began on a laboratory scale under the direction of N. W. Pirie more than thirty years ago. Units developed at the Rothamsted Experimental Station handle one to two tons of leaf crop per hour (21) and similar machines have been distributed to various countries.

Feeding trials conducted by various workers have shown that leaf protein concentrates are of high nutritional value, and without deleterious components. Duckworth and co-workers (23) fed pigs, Henry and Ford (31) fed rats and Waterlow (71) supplemented the diets of infants. These workers concur in giving leaf protein that has not been damaged through drying by inappropriate methods, a value as great as that of fish meal or the best seed protein, but not so good as casein or egg protein.

Another consideration in the development of protein sources is the matter of acceptability by the populace for whom they are designed. The high chlorophyll content would doubtlessly limit the adoption of

leaf protein concentrates as a main protein dish. Rather, the more practical approach would appear to be to supplement the customary diets with amounts to enhance the protein quality yet not alter noticeably the flavor of familiar dishes.

It is recognized that those protein-deficient people who presently subsist largely on cereals are receiving less than optimal intakes of lysine and threonine. The amino acid composition (29) of leaf protein concentrates indicates that these proteins are fairly well balanced except for methionine and the amino acid composition varies little for different types of leaf. The suitability of leaf protein concentrates for the supplementation of cereal-based diets low in lysine is thus apparent.

In view of the interest in our laboratory in testing the technique of microbiological assay of protein quality, it was decided to use the protozoan Tetrahymena pyriformis W as the test organism. This ciliate possesses strongly proteolytic enzymes (36) and can utilize intact protein (4). It is particularly suited for the evaluation of protein quality intended for human use because it has an absolute requirement for the ten essential amino acids required by higher animals (36). Studies have shown that it exhibits a sensitivity to heat damaged proteins (69).

The purpose of the present investigation was twofold. The main aim was to evaluate the protein quality of a leaf concentrate in order to determine its suitability for supplementation of primarily cereal diets. The secondary aim was to test the suitability of the use of the protozoan, Tetrahymena pyriformis W for routine measurement of protein quality.

REVIEW OF LITERATURE

Conventional Methods of Protein Evaluation

A number of methods have been devised to measure the nutritive value of proteins. One of the ones most commonly used is the determination of protein efficiency ratio (PER) introduced by Osborne, Mendel and Ferry in 1919 (52). In this method a protein is fed to a weanling animal for a given period of time. The grams of body weight gained during that time, divided by the grams of protein consumed, gives the protein efficiency ratio. Block and Mitchell (8) after an extensive review of the data point out that it is not a true efficiency ratio since not all the protein is used for growth and not all the weight gained is protein.

Bioassay methods include nitrogen balance studies (38), growth studies (34) and measurement of weight regain in animals previously subjected to protein depletion as developed by Cannon (15).

Mitchell (46) proposed another method of protein evaluation. It is the determination of biological value (BV) in terms of per cent of absorbed nitrogen retained by a growing animal. The product of the coefficient of digestibility and biological value represents the proportion of food nitrogen retained and is expressed by the single index called Net Protein Utilization (NPU).

The nitrogen balance index of Allison (2) is essentially the same as biological value. It is nitrogen balance minus nitrogen balance when nitrogen intake is zero, divided by absorbed nitrogen. This index is a measure of dietary nitrogen retained.

The net protein utilization (NPU) of Miller and Bender (44) expresses both the digestibility and biological value of a protein. When used to compare the quality of proteins, NPU is measured under standardized conditions. The protein is supplied at maintenance levels in a diet that provides adequate calories. (NPU_{op}) net protein utilization operative (45) refers to the utilization of a protein under the conditions in which it is consumed. The (ND_pV) net dietary protein value (58) is the product of protein concentration and net protein utilization and is a measure of the efficiency and concentration of a protein.

Bender and Doell (7) extended the PER calculation to include an allowance for maintenance. They did this by adding to the weight gain in the PER calculation, the weight lost by a group of rats fed a protein-free diet. The new calculation was called the net protein ratio (NPR) and when multiplied by the actual per cent of protein in the carcass, the protein retention efficiency (PRE) was obtained. Thus the PRE corresponds closely to NPU assuming that there are no important differences in the protein content of the carcass.

Jansen (35) reported the total protein value of protein - andamino acid-supplemented bread. In this method the PRE or quality factor was multiplied by the quantity factor, the fraction of protein in the bread and the resulting value was called net protein value per cent.

Almquist (3) and Carpenter and co-workers (18) have used the gross protein value (GPV) to measure the value of a protein source as a supplement to cereals.

The above methods illustrate the use of protein evaluation methods

in the animals for which they are intended or when this is not possible in animals that closely resemble the species for which the protein is to be evaluated.

However, these methods involve relatively long-term studies. In vitro methods of protein evaluation save time and expense. Modern methods of amino acid analysis have assisted in the development of reliable in vitro procedures for measurement of protein quality (48) (53). The data from these methods correlate reasonably well with biological values. They also indicate the limiting essential amino acids so that adequate supplementation can be made. However, the biological values of most proteins are changed by heat processing making knowledge of amino acid availability necessary. A variety of procedures have been developed to measure amino acid availability in vitro (40) (17).

Sheffner, Eckfeldt and Spector (62) developed an integrated index which combines the pattern of amino acids released by in vitro pepsin digestion with the amino acid pattern of the remainder of the protein. This new index known as the pepsin digest residue (PDR) index correlates closely with the net protein utilization of a variety of proteins and detects changes in heat treated proteins as well (63). Akeson and Stahmann (1) used the PDR index substituting ion-exchange chromatography for microbiological analysis of amino acids.

Enzymatic methods of protein evaluation have received increased attention in recent years. Melnick, Oser and Weiss (42) proposed that the rate of release of amino acids from protein by pancreatin digestion as well as total amino acid composition, was an important factor in the

nutritional quality of protein. Mauron and co-workers (40) (41) using *in vitro* digestion of proteins with pepsin followed by pancreatin, measured the digestibility and availability of lysine.

Another well used method for evaluation of proteins is the chemical score of Mitchell and Block (48). It predicts the nutritive value of a protein based on comparison of the essential amino acid content of a test protein with a standard. Egg protein, assigned a biological value of 100, has been used as a standard. This method is based on the relative amount of the essential amino acid in greatest deficit when compared to egg.

A number of amino acid indices have been used to estimate protein value. The Kuhnau Integrated Index (37) rates the proteins according to the sum of the percentages of essential amino acids. The Oser Essential Amino Acid Index (53) uses certain features of Kuhnau's index and the egg ratio concept of Mitchell and Block. The Modified Essential Amino Acid Index of Mitchell (47) gives values which correlate closely with biological values for the growing rat. These indices do not take into consideration the effects of heat processing on the subsequent liberation of amino acids during digestion.

The FAO pattern (73) has been also used as a standard. This method was proposed by the 1963 Joint Food and Agriculture Organization/World Health Organization Expert Group. Bressani and co-workers (10) (11) (12) in their studies with children have used this FAO reference pattern as the basis for supplementation of corn and wheat. Their results suggested that the FAO requirement for both sulfur-containing amino acids and tryptophan may be too high, since reducing the total proportion of sulfur

amino acids to tryptophan in corn-supplemented diets had no detrimental effects on nitrogen balance. When the FAO pattern is corrected for these high values, it more closely resembles the pattern for whole egg and human milk. The FAO/WHO group (51) also recommended that in the amino acid reference pattern the relationship of each essential amino acid to the total of essential amino acids be used.

Various chemical methods provide a rapid analysis of protein quality. The Kjeldahl method measures total nitrogen but gives no indication of the quality of the protein. For soybean protein, protein solubility tests of Evans (25) have been applied and urease inactivation has been used as a test for inadequate heating.

Microbiological Methods

Microbiological methods have a considerable potential for the evaluation of protein quality. They offer possibilities of greater economy in time, money, labour and materials and because of their rapid three to four day test period procedures, large numbers of samples can be screened for protein quality. They serve a double role in that they measure either the overall nutritive value of protein or the availability of individual essential amino acids according to the technique used.

Interest has developed in the use of various strains of bacteria including Streptococcus faecalis, Streptococcus zymogenes, Leuconostoc mesenteroides, Pseudomonas aeruginosa, and the protozoan, Tetrahymena pyriformis.

Streptococcus faecalis. For assays with Streptococcus faecalis, Halevy and Grossowicz (30) hydrolyzed a variety of proteins for forty-eight hours with pancreatin and determined the amount of hydrolysates needed

for half-maximum growth in their test cultures. The organism correctly indicated that the nutritive value of egg albumen was greater than that of casein but not to the extent shown by assays with rats. This organism, however, indicated that the limiting amino acid for egg albumen and casein was lysine whereas the limiting amino acid for egg albumen actually is isoleucine and for casein is methionine plus cystine. This suggests that S. faecalis requires more lysine than does the growing or depleted rat.

Teeri and co-workers (68) used both Streptococcus faecalis and the enzymes, pepsin, pancreatin and erepsin to hydrolyze the test protein. They found that for better proteins there was good agreement with the net protein utilization values but the same did not hold true for poor quality proteins. The shortcoming of this technique was that Streptococcus faecalis has a requirement for only a few amino acids. This limits the evaluation to a few deficient amino acids. Bunyan and Price (13) using Streptococcus faecalis, were not able to distinguish between a variety of meat meals which ranged in biological value from one-third to two-thirds that of casein.

Streptococcus zymogenes. Ford (27) used the proteolytic bacterium, Streptococcus zymogenes to measure the relative nutritive value (RNV) of a variety of food proteins. Relative nutritive value was defined as the amount of growth that occurs on a protein food compared with the amount of growth on casein at a similar nitrogen level under the same conditions. The casein was given an arbitrary value of 100. The values obtained were found to correlate closely with those obtained in biological

tests with rats. This organism is potentially useful for measuring certain individual amino acids as methionine, tryptophan, arginine, histidine, leucine, isoleucine, valine and glutamic acid, both free and in intact proteins. Growth response was measured by (1) turbidity (2) reduction of triphenyltetrazolium chloride (3) measurement of titratable acidity and lactic acid production. Values obtained for several meat meals were closely correlated with their content of available lysine. Streptococcus zymogenes is vigorously proteolytic but pretreatment of the casein and test sample with papain improved the assay by speeding growth. The availability of amino acids in various proteins for growth of Streptococcus zymogenes has been studied by Waterworth (72) and Ford (28).

Leuconostoc mesenteroides. Horn, Blum, Womack and Gersdoff (32) and Horn, Blum and Womack (33) used Leuconostoc mesenteroides P-60 to grade a number of cottonseed meals processed under different conditions. The meals were incubated with trypsin, pepsin, and a preparation of pig-gut mucosa for successive twenty-four hour periods. The growth of the organism with a digest of a reference 'standard' meal was obtained and comparisons made with growth of the organism on the above enzymic digests. These workers then computed 'indices of protein value' which agreed fairly well with the findings in rat growth tests.

Pseudomonas aeruginosa. A bacterial method for measuring protein digestibility was recommended by Mertz and co-workers (43) using the strongly proteolytic Pseudomonas aeruginosa. The results were in general agreement with those found in tests with rats.

Tetrahymena pyriformis. The comprehensive studies of Kidder and

Dewey (36) on the nutritional requirements of the protozoan Tetrahymena pyriformis have revealed the potentialities of this organism as an experimental animal. It is a ciliated protozoan which can be grown in axenic culture on a chemically defined medium and its nutritional requirements correspond to what we know of higher animals. The W strain requires absolutely arginine, histidine, leucine, isoleucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine, the ten amino acids regarded as essential for the growth of higher animals.

The most important features of this species (strain W) as an organism for evaluating protein quality are its requirement for the above amino acids and its ability to utilize intact protein. Hydrolysis of the test sample is not a necessary preliminary to the assays and tests with this organism simulate more closely the circumstances of the biological test using higher animals.

Dunn and Rockland (24) (60) used Tetrahymena pyriformis H to assay protein quality. They used acid production as an index but their assay lasted forty-one days.

Anderson and Williams (4) proposed a colorimetric method for the determination of growth of Tetrahymena pyriformis W. Growth responses were measured by determination of the red triphenylformazan (TPF) formed by the enzymatic reduction of colourless 2,3,5 triphenyltetrazolium chloride (TPTZ). An incubation period of three to five days was required for this procedure which permitted a more extensive evaluation of the growth requirements of the organism. However, there was poor correlation between the response of the organism to various proteins and values

observed with the growing rat.

Pilcher and Williams (54) used a modification of the TPTZ method for measuring growth of Tetrahymena pyriformis W. They found substantial differences in the relative nutritive values of egg and ground nut protein between three and five-day assays due probably to their use of a dye reduction response to estimate growth. This was later demonstrated to be unsatisfactory by Fernell and Rosen (26).

Fernell and Rosen (26) further modified the above procedure. Whereas Pilcher and Williams (54) used growth in relation to food nitrogen to compare proteins, they used growth in relation to ammonia nitrogen production to test the efficiency of protein utilization. These investigators found that when various proteins were added to the culture in the presence of TPTZ, the colours produced varied according to the nature of the protein added. Consequently, they assessed growth response by direct microscopic count.

Fernell and Rosen (61) using Tetrahymena pyriformis W to assess the relative nutritive value of protein materials obtained values that were in general agreement with the chemical scores and protein efficiency ratios (PER) for the rat. However, net protein utilization was not as well correlated. Nevertheless, these workers were able to demonstrate a lowering in the nutritive value of groundnut and soya bean protein resulting from overheating. The beneficial effect of tryptophan supplementation of gelatin was also observed.

Viswanatha and Liener (70) have shown that denaturation of soybean, serum albumin and egg albumin increased the ability of the protozoan

to utilize these proteins.

Stott and Smith (65) determined the available lysine, methionine, arginine, and histidine in animal protein sources and vegetable sources. Values obtained for animal protein sources were similar to those obtained by Carpenter for lysine using the fluoro - 2,4 - dinitrobenzene (FDNB) method for samples with higher available lysine content. There was a tendency for Tetrahymena values to be lower in samples with lower available lysine content. There was little variation for groundnut meals and soya bean meals between samples of one type of oilseed meal, but considerable variation between samples in the available lysine content of cottonseed meals. The use of Tetrahymena pyriformis W for assay of available isoleucine, leucine, phenylalanine, threonine, tryptophan and valine in addition to the four used in this study is suggested by these authors (65).

Teunisson (69) showed that different proteins gave a maximum growth response at different nitrogen levels and had a characteristic growth response curve.

Stott, Smith and Rosen (66) proposed a simplified assay procedure in which organism count after four days' incubation was used as a simple criterion of the nutritive value of protein. Typical improvements in the nutritive value of certain vegetable proteins when supplemented with methionine and lysine were observed.

Plant Protein Sources

It has been well established that a majority of the world's population subsists on cereal diets that are deficient in protein.

Simply intensifying agriculture will increase this supply of low quality protein foods, rather than improve the concentration of protein needed. Animal products used extensively in the developed countries are economically impractical in other parts of the world.

Leaf protein. N. W. Pirie (56) has pioneered the production of concentrates from leaves. Fresh material in which the protein is undenatured and soluble is pulped and pressed, the extracted protein is then coagulated, filtered off and washed. Several procedures for making leaf protein concentrates on a large scale and for drying and storing have been described (50) (55) (22). The basic product is a dark green presscake containing about sixty per cent water, with little or no smell or taste. It has the consistency of cheese or yeast and putrified easily at room temperature.

Subba Rao (67) has described a method of preservation for wet leaf protein concentrates. These can be dried by a variety of methods without changing the nutritive value of the protein. Drying methods are freeze drying, extraction with acetone followed by vacuum drying at 30°C and mixing the leaf concentrate with dry barley flour followed by drying in a stream of air heated to 40°C. Duckworth (22) showed that heating above 84°C drastically reduced the gross protein value.

N. W. Pirie (57) has visualized various sources of leaf for protein production. In different countries, it is convenient to make protein from different species of leaf. Chibnall, Rees and Lugg (19) reported that proteins extracted by various methods from green plants showed only slight differences in amino acid composition. Pleshkow and Fowden (59)

and Gerloff and co-workers (29) analyzed leaf protein concentrates for their amino acid composition and found that preparations from leaves of different species and ages were similar.

The value of the protein as a supplement in the diets of weanling pigs has been shown by Barber, Braude and Mitchell (6). Duckworth, Hepburn and Woodham (23) showed that leaf protein was effective as a supplement to a predominantly barley meal for pigs. Assays of the biological value of leaf protein concentrates were carried out with chicks and rats (22). The nutritive value of these concentrates ranged from a biological value of seventy-one to eighty-two while that for soybean meal was seventy-four. Waterlow (71) demonstrated that there was nearly as good nitrogen retention by malnourished infants fed a mixture of leaf protein plus milk as on milk alone fed at equal protein levels. These studies indicate that leaf protein could be used to supplement human diets and help combat protein malnutrition. The presentation of leaf protein is further discussed by Byers (14), Pirie (56) and Morrison (49). In countries with largely vegetarian diets such as New Guinea, Nigeria and South India, the green colour is not objectionable (57).

The advantage of making leaf protein concentrate is that a greater yield can be obtained than with seeds, tubers or by animal conversion which is inefficient (N. W. Pirie, personal communication). The present day need is for more skill in making protein from local materials, not for protein exported by industrialized countries (56).

EXPERIMENTAL

Test Organism

The test organism, *Tetrahymena pyriformis W* #10542, was obtained from the American Type Culture Collection, Rockville, Maryland. This organism has been described in the literature as *Tetrahymena pyriformis*, *T. geleii*, *Glaucoma pyriformis*. The nomenclature and status of strains of the Colpidium-Glaucoma-Leucophrys-Tetrahymena group of holotrichs have been reviewed by Corliss (20).

Maintenance. Axenic stock cultures were maintained at $25^{\circ} \pm 1^{\circ}C$ in 125 ml. opaque flasks containing 20 ml. of nutrient broth of the following composition (% w/v): proteose peptone (Difco) 0.5, Bacto-tryptone (Difco) 0.5, potassium phosphate (monobasic) 0.02, adjusted to pH 7.1 and sterilized by autoclaving[1] at $121^{\circ}C$ for ten minutes. Stock cultures were transferred at approximately weekly intervals to ensure a live healthy culture at all times. Daily transfers were made to ensure a supply of organisms of a suitable age for inoculation each day. Cultures were checked periodically for contamination by plating on nutrient agar.

Basal medium. Table I shows the composition of the basal medium. It was derived from the D medium of Kidder and Dewey (36). Stock solutions, A, B, C, D, all 100 times as concentrated as in the final medium were kept of these groups of components, with the exception of group E, which was weighed out as required (Table II).

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[1] Castle 999-C

TABLE I

COMPOSITION OF BASAL MEDIUM

	Component	Concentration in Assay Medium (mcg/ml.)
(A)	Calcium pantothenate	0.625
	Nicotinamide	0.625
	Pyridoxine hydrochloride	6.25
	Pyridoxal hydrochloride	0.625
	Pyridoxamine hydrochloride	0.625
	Riboflavin	0.625
	Folic acid	0.0625
	Thiamine hydrochloride	6.25
	Inositol	0.625
	Choline chloride	6.25
	p-Aminobenzoic acid	0.625
	Biotin	0.0625
	DL-α-lipoic acid	0.02
(B)	$MgSO_4.7H_2O$	140.0
	$FE(NH_4)_2(SO_4)_2.6H_2O$	62.5
	$MnCl_2.4H_2O$	1.25
	$ZnCl_2$	0.125
(C)	$CaCl_2.2H_2O$	30.0
	$CuCl_2.2H_2O$	3.0
	$FeCl_3.6H_2O$	0.75
(D)	K_2HPO_4	175.0
	KH_2PO_4	175.0
(E)	Guanylic acid	150.0
	Adenosine 3'(2') phosphoric acid	100.0
	Cytidylic acid	125.0
	Uracil	50.0
(F)	Glucose	4,000.0

TABLE II

COMPOSITION OF STOCK SOLUTIONS AND SOLUTION E

Stock solution A (100 times final strength)	(mg/200 ml.)
Calcium pantothenate	12.5
Nicotinamide	12.5
Pyridoxine hydrochloride	125.0
Pyridoxal hydrochloride	12.5
Pyridoxamine hydrochloride	12.5
Riboflavin	12.5
Folic acid	1.25
Thiamine hydrochloride	125.0
Inositol	12.5
Choline chloride	125.0
p-Aminobenzoic acid	12.5
DL-α-lipoic acid	0.4
Biotin	1.25
Stock solution B (100 times final strength)	**(g/200 ml.)**
$MgSO_4.7H_2O$	2.8
$Fe(NH_4)_2(SO_4)_2.6H_2O$	1.25
$MnCl_2.4H_2O$	0.025
$ZnCl_2$	0.0025
Stock solution C (100 times final strength)	**(mg/200 ml.)**
$CaCl_2.2H_2O$	600.0
$CuCl_2.2H_2O$	60.0
$FeCl_3.6H_2O$	15.0
Stock solution D (100 times final strength)	**(g/200 ml.)**
KH_2PO_4	3.5
K_2HPO_4	3.5
Solution E (5 times final strength)	**(mg/20 ml.)**
Guanylic acid	15.0
Adenosine-3'(2')-phosphoric acid	10.0
Cytidylic acid	12.5
Uracil	5.0

Solution E, of five times the final concentration was prepared from stock solutions B, C, D and component E by dissolving the appropriate weights of component E (Table II) in approximately 10 ml. distilled water. 1 ml. each of stock solutions B, C, D was added and the whole made up to 20 ml. This was adjusted to pH 8.2 with 0.5 N NaOH. In earlier work (26), the pH used was 7.1 but 8.2 was adopted by Stott, Smith and Rosen (66) to eliminate the tendency for the pH to drop below 7.1 when solution E plus the protein was autoclaved.

Protein Sources

The reference standard used was vitamin-free casein which was obtained from Nutritional Biochemicals Corporation. This casein was adopted as reference because it is a purified protein of high biological value and is commonly used as a control in feeding tests of higher animals and in other microbiological assays.

Leaf protein. The plant Chenopodium quinoa was used as the source of leaf protein. Plants were cultivated under controlled conditions in the greenhouse of the Plant Science Department, University of Manitoba, at a temperature of 78°F on clay loam soil.

The plants were harvested when six weeks old and approximately 14 inches high. At this stage, the leaves were not fully mature and thus relatively tender. Young leaves are desirable since the amount of protein that can be obtained from the leaves decreases with maturity.

The leaf protein concentrate was prepared according to the method of Morrison and Pirie (50). Fresh leaves were pulped in a household Waring blendor with the addition of water. Two extractions were made. The pulp was then strained through cotton to remove the residual fibre.

The pH of the liquid was adjusted to 3-4 and coagulated with steam. The coagulated material was filtered and suspended in water (pH 4.0), filtered again and pressed to 20% dry matter. The acid pH increases the keeping quality of the concentrate and suspending in water serves as a 'washing' step.

Portions of the moist press-cake were then quick frozen in a household freezer, freeze-dried, decolorized, oven-dried, or refrigerated. The freeze-dried and decolorized samples were prepared by the Food Science Department, University of Manitoba. The press-cake was frozen in liquid nitrogen and placed in the vacuum chamber of a freeze dryer[2] until dry. The decolorized samples were prepared by Soxhlet extraction of the leaf protein with acetone. The sample was air dried in an open petri dish, then placed in a vacuum dessicator (10 hrs.) to remove all acetone traces. The oven-dried samples were placed in an oven[3] at 65°C for four hours. The freeze-dried, decolorized and oven-dried samples were kept in tightly closed containers at room temperature. Of the three dried samples, the freeze-dried was the most susceptible to oxidation.

Moisture determinations on the freeze-dried and decolorized samples were made by the Food Science Department at the time of preparation of the above samples. All other determinations were made by the Air Oven method (5).

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[2] Virtis

[3] Thelco oven

The nitrogen content of the test materials was determined by the Kjeldahl method and crude protein content was calculated by multiplying nitrogen by 6.25. Amino acid analyses were determined for all leaf protein samples using a Beckman Model 116 Amino acid analyzer.

Quantity of protein used. In previously reported studies using Tetrahymena pyriformis W, the level of nitrogen at which test proteins were compared was shown to be very important. Fernell and Rosen (26) found that at high nitrogen levels, poorer quality protein such as soybean and groundnut, gave increasingly higher values while for vitamin-free casein there was little increase. Anderson and Williams (4) reported a similar cross-over of response. Relative nutritive values therefore depend markedly on the nitrogen level selected for comparison. Rosen and Fernell (16) found ground nut germ to be superior to casein for 0 to 3 mg.N/ml but inferior for 0 to 1 mg.N/ml. In this study, the level of 0.5 mg.N/ml. was chosen in order to give a more accurate picture of the growth promoting abilities of the various leaf protein concentrates.

The amount of protein supplied by the vitamin-free casein and the various treatments of leaf protein is given below in percentages:

Vitamin-free casein	- 90.6%
Miost press-cake	- 65.0%
Frozen	- 65.0%
Freeze-dried	- 58.38%
Decolorized	- 69.69%
Oven dried	- 40.0%

Supplementation with crystalline amino acids. Calculation of the

Chemical Score of Block and Mitchell (8) gave the chemical scores shown in Table III using the essential amino acid values for whole egg. This served as a useful screening procedure to quantitate biological values. On the basis of the amino acid composition given in Table III, the first and second limiting amino acids appeared to be methionine and isoleucine. Lysine, although not significantly lower than in casein, was chosen because of its susceptibility to heat damage.

For each leaf treatment, the three amino acids were added singly and in all combinations in amounts to bring them up to the level of vitamin-free casein (Table IV). The addition of amino acids within each group was completely randomized.

Preparation of samples. The procedure followed in this study was essentially that of Stott, Smith and Rosen (66). All leaf protein samples at a level of 0.5 mg.N/ml. were wetted and ground to a smooth paste in a mortar and pestle. Distilled water was then added to bring it to volume and the pH was adjusted to 8.2 with 0.5 N NaOH. Dispersions were allowed to stand overnight in the refrigerator in order to minimize changes in pH on subsequent autoclaving. After warming to room temperature, the pH was readjusted to 8.2.

The vitamin-free casein suspension was prepared by mixing 0.5 N NaOH with the casein (at a level of 0.5 mg.N/ml.) to form a smooth paste. This was transferred to a flask, a small amount of distilled water was added and the flask agitated to stir the mixture. The resulting suspension was brought to volume with distilled water and the pH adjusted to 8.2.

TABLE III

AMINO ACID COMPOSITION OF TEST PROTEINS (g/16g N)

Amino Acids	V.F. Casein*	Moist Press-Cake	Decolorized Leaf Protein	Freeze-Dried Leaf Protein	Oven-Dried Leaf Protein
Lys[x]	8.0	7.15	6.41	6.27	6.0
His	3.1	2.80	2.58	2.95	2.38
Arg	7.3	8.25	6.26	6.16	6.04
Asp	3.6	11.83	9.61	9.73	9.28
Thr	4.3	5.87	4.84	4.86	4.64
Ser		5.13	4.44	4.30	4.32
Glu	23.0	17.05	11.30	11.30	13.73
Pro		5.80	4.78	3.98	4.78
Gly	2.0	7.96	5.72	5.70	6.45
Ala		6.10	6.34	6.25	4.98
Cys		0.51	0.41	0.42	0.33
Val	7.3	6.42	5.56	5.57	4.98
Met	3.3	1.50	1.80	1.08	0.94
Ileu	6.0	5.27	4.54	4.59	4.37
Leu	10.0	10.96	9.0	9.09	9.03
Tyr		5.73	5.03	5.01	4.47
Phe	5.4	7.10	6.03	5.87	5.75
Chemical Score[+](8) 62[']		38	41	28	23

[*] From J. Block and K. W. Weiss, Amino Acid Handbook (Charles C. Thomas, Illinois, 1956), p. 266.

[+] Chemical Score of Block and Mitchell using whole egg ratios.

[']From A. A. Albanese, Newer Methods of Nutritional Biochemistry (Vol. III; Academic Press, 1967), p. 131.

[x] Lys, Lysine; His, Histidine; Arg, Arginine; Asp, Aspartic Acid; Thr, Threonine; Ser, Serine; Glu, Glutamic Acid; Pro, Proline; Gly, Glycine Ala, Alanine; Cys, Cystine; Val, Valine; Met, Methionine; Ileu, Isoleucine; Leu, Leucine; Tyr, Tyrosine; Phe, Phenylalanine.

TABLE IV

SUPPLEMENTATION WITH AMINO ACIDS

Supplement	Protein Source				
	Moist Cake g/16g N	Quick Frozen g/16g N	Decolorized g/16g N	Freeze-Dried g/16g N	Oven-Dried g/16g N
DL-Met	1.80	1.80	1.50	2.22	2.36
L-Ileu	0.73	0.73	1.46	1.41	1.63
L-Lys	0.85	0.85	1.59	1.73	2.0
L-Ileu+	0.73	0.73	1.46	1.41	1.63
DL-Met	1.80	1.80	1.50	2.22	2.36
L-Ileu+	0.73	0.73	1.46	1.41	1.63
L-Lys	0.85	0.85	1.59	1.73	2.0
L-Lys	0.85	0.85	1.59	1.73	2.0
DL-Met	1.80	1.80	1.50	2.22	2.36
L-Ileu+	0.73	0.73	1.46	1.41	1.63
L-Lys+	0.85	0.85	1.59	1.73	2.0
DL-Met	1.80	1.80	1.50	2.22	2.36

All pH measurements were made using a Corning Model 5 pH meter.

Assay Procedure

A total volume of 10 ml. in a 2-ounce brown bottle was used made up of the following adjusted to pH 8.2:

(a) 2 ml. of solution E (Table II)

(b) 4 ml. of protein suspension 2.5 times the final strength

(c) 2 ml. of distilled water

The bottles were then autoclaved at 121°C for ten minutes. Two solutions were autoclaved separately at 121°C for ten minutes: (a) the stock vitamin solution A (Table II) diluted ten times; (b) a solution containing 4% (w/v) glucose. As a preliminary part of the work now described, the glucose at levels of 15% as described by Rosen and co-workers (61) and 7.5% were used. By the second day the medium was relatively acid (pH 5.4) and on the fourth day the pH had dropped to 4.5 and the organisms failed to survive. The level of 4% glucose maintained healthy active organisms both in the leaf protein suspension and in the vitamin-free casein.

To each bottle after cooling were added aseptically 1 ml. diluted solution A and 1 ml. glucose solution. Each bottle was inoculated with three drops of a 3-day broth culture of *T. pyriformis W*, and incubated at 25°±1°C for 4 days. Cotton plugs were used and the bottles were inclined in special racks at 15° to the horizontal to provide ample aeration for rapid growth.

After 4 days' incubation, the bottles were removed for analysis. Cultures grown in duplicate were combined for analysis. The cultures

were shaken and the contents filtered coarsely with glass wool to remove the leaf residues. They were then transferred to a modified separatory funnel designed with a raised outlet $\frac{3}{4}$ inches high and a bore hole at the top of the outlet tube, 0.5 mm. The raised outlet permitted only motile organisms to be released when the stopcock was opened.

One ml. of the culture was transferred to a screw-cap Kimax test tube containing 1 ml. preserving fluid. This fluid consisted of 90 ml. water, 20 ml. 40% formaldehyde and 10 ml. stock solution D (Table II). Because of the extreme difficulty encountered in distinguishing the organisms in the dark green leaf solution, 3 drops of iodine were added to bring the preserving fluid to 1 ml. The iodine stains the cytoplasmic granules a bright orange. In preparing the material for counting the organisms, it was found necessary to agitate the test tube containing the preserving fluid while adding the culture to avoid clumping of the organisms.

The number of organisms was determined by direct microscopic count in a Spencer, improved Neubauer, double-cell haemocytometer (0.1 mm. deep). The organisms in 9 sq. mm. areas of the network of each cell were counted and the mean number per 1 mm. square, multiplied by the dilution, gave the final population of the test culture in units of 10^4 organisms per milliliter of culture. Two operators made separate counts on each sample. The count method was chosen in preference to the conventional turbidity and colorimetric procedures because of the insoluble and coloured materials in the medium. A binocular microscope[4] on low

[4]Olympus microscope, model EH

power was used to obtain adequate resolutions. Assays were made in triplicate for each treatment of leaf.

Analysis of results. The supplementation of amino acids was designed as a 2^3 factorial experiment and analyzed in a 2^3 factorial form in conjunction with the leaf processes. Analysis of variance as outlined by Steel and Torrie (64) was applied to the data. Relative nutritive values (RNV's) were determined for all leaf protein concentrates using casein as 100.

RESULTS AND DISCUSSION

Amino Acid Composition and Protein Quality

The nutritional value or ability of protein to replace body nitrogen depends on the composition and availability of its essential amino acids. Table III gives the amino acid composition of various treatments of leaf protein and of vitamin-free casein. In general, all treatments had similar amino acid composition when expressed as percentage of protein. The composition of the moist cake compared favorably with literature values reported for other leaf protein concentrates (29). When compared with casein, methionine, isoleucine and lysine were lower in all treatments but threonine and phenylalanine were consistently higher. In general, it appeared that essential amino acids of the treated samples were slightly decreased below those present in the moist press-cake.

The Committee on Protein Malnutrition (51) has recommended the use of total essential amino acids as a percent of total nitrogen as a determining factor of the nutritive value of proteins. Two proteins may vary in quality because of differences in the content of essential amino acids per 100 grams of total nitrogen. For example, calculation of the total nitrogen supplied by the essential amino acids of such high quality proteins as egg or milk shows them to have 35% or more, whereas values of 24% or less are assigned to such cereal grains as barley and wheat. Comparable values for the leaf protein concentrates tested in this study are shown:

Moist press-cake	44.3%
Decolorized leaf protein	38.2%

Freeze-dried leaf protein	37.3%
Oven-dried leaf protein	36.0%

Barber and co-workers (6) reported that leaf protein concentrate was nutritionally equal to white fish meal when fed to pigs and Duckworth (22) found the gross protein value (GPV) of leaf protein concentrate to be higher than soybean meal. A comparison of the aminograms of the leaf protein concentrates used in this study, with those of soybean meal and fish meal (Table V) revealed that methionine was lower in the leaf protein concentrate than in soybean meal and in fish meal, but that lysine was higher in the leaf protein preparation than it was in the soybean meal.

Growth response of Tetrahymena pyriformis W on casein and unsupplemented leaf protein treatments.

Amino acid composition alone does not determine the nutritive value of a foodstuff. Amino acid levels measured after acid hydrolysis of foods, roughly predict their potential value in nutrition, but for some materials the effective value is significantly less. The rate and degree of release of amino acids during digestion are important determinants of protein quality. Tetrahymena pyriformis W possesses strongly proteolytic enzymes and can digest protein effectively (36). Vitamin-free casein, the reference protein, when fed at the level of 0.5 mg. N/ml. of medium, supported a mean growth of 0.55 million organisms/ml. Little work has been reported for the growth of T. pyriformis W on leaf protein concentrates and what has been published does not indicate the number of organisms per milliliter of culture. Rosen and Fernell (61) reported that the organism failed to grow on grass-protein concentrates perhaps owing to

TABLE V

A COMPARISON OF THE ESSENTIAL AMINO ACID CONTENT (g/16g N)
OF LEAF PROTEIN WITH OTHER FOODSTUFFS

Amino Acids	L.P.C.[a]	Fishmeal[b]	Soybean meal[b]
Lys	7.15	10.4	6.2
Phe	7.10	4.2	4.8
Met	1.50	3.0	1.7
Thr	5.87	4.6	3.9
Leu	10.96	8.4	8.2
Ileu	5.27	6.0	6.4
Val	6.42	5.7	5.0
Trp	---	1.1	1.4

[a]Moist press-cake

[b]From F. B. Morrison, Feeds and Feeding (Morrison Publishing Company, New York, 1965), p. 1165.

the extreme insolubility of these products at pH 7.1.

The results given in Table VI show that counts of organisms per milliliter were extremely low for the unsupplemented leaf proteins when compared with vitamin-free casein. At the same time it is of interest to note that the decolorized sample supported much better growth than any of the other unsupplemented leaf processes. This may have been due to the fact that the acetone-soluble lipid material present in the leaf and removed by the decolorizing process proved to be inhibitory to the growth of the organism. Kidder and Dewey (36) have shown that a number of unsaturated fatty acids inhibit the growth of Tetrahymena pyriformis W. Analyses of fatty acid components of leaf protein concentrates by Lima and co-workers (39) show that the principal fatty acids in leaf protein concentrates are linoleic, palmitic and linolenic acids. Chenopodium amaranticolor, one of the leaf proteins tested in that study, contained a higher proportion of oleate than the other concentrates, in addition to the unsaturated fatty acids present in other leaves. The chlorophyll breakdown products that were present may also have been partially responsible for the results in the green leaf preparations.

Oven-drying at 65°C for four hours resulted in an extremely brittle product which resisted grinding. Duckworth and Woodham (22) reported that slow drying of leaf protein in a current of air caused a hard, brittle product and suggested drying in the presence of barley flour. This was not attempted however, as it was the intention of this experimenter to test leaf protein without the addition of any other material. Amino acid analysis of the oven dried sample (Table III) showed that heat damage to

TABLE VI

COMPARISON OF GROWTH OF *TETRAHYMENA PYRIFORMIS* W OBTAINED WITH CASEIN AND UNSUPPLEMENTED LEAF PROTEIN TREATMENTS

Protein Source	Counts (10^4/ml.)
Vitamin-free casein	55.0
Moist press-cake	15.3
Frozen leaf protein	12.0
Freeze-dried leaf protein	16.6
Decolorized leaf protein	25.6
Oven-dried leaf protein	2.0

the essential amino acids was not more severe than the other processes. This is in accord with the findings of Duckworth and Woodham (22) that only drying above 80°C markedly reduced the nutritive value of leaf protein concentrates for chicks and growing rats. The extremely poor growth of Tetrahymena pyriformis W on the oven dried sample may be attributed to the inability of the proteolytic enzymes to act on this insoluble material. Values reported here are therefore not truly representative and cannot be compared with oven-dried preparations tested by more conventional methods, and for this reason have been excluded from the statistical analysis.

Effect of supplementation by crystalline amino acids

The limiting amino acid in a food depends on the quantitative requirements of the organism and its ability to extract that amino acid from the food. Thus the amino acid present in least amount relative to the demands of the organism, is usually the one that limits growth. From the amino acid analysis (Table III) and comparison with the high quality reference protein, casein, methionine was determined to be the first limiting amino acid for all leaf protein treatments. Amounts of crystalline DL-methionine were added to each treatment to bring the level of methionine in each case to that of vitamin-free casein. Similarly, all leaf protein treatments were supplemented with L-isoleucine and L-lysine.

The treatment means for the different processes and supplements are given in Table VII. The analysis of variance of the data (Table VIII) showed that the main effects, processes and amino acids, were

TABLE VII

THE EFFECT OF SUPPLEMENTATION OF LEAF PROTEIN WITH CRYSTALLINE AMINO ACIDS ON GROWTH OF TETRAHYMENA PYRIFORMIS W

Protein Source	Counts (10^4/ml.)						
	Met	Ileu	Lys	Ileu + Met	Ileu + Lys	Lys + Met	Ileu + Lys Met
Moist press-cake	40.0	18.0	25.3	28.0	22.0	41.3	39.3
Frozen leaf protein	30.0	18.0	16.6	19.3	18.0	34.0	29.3
Freeze-dried leaf protein	41.3	18.6	30.6	22.3	20.6	35.3	38.0
Decolorized leaf protein	40.0	29.3	42.6	25.3	27.0	52.0	49.3
Oven-dried leaf protein	3.3	8.6	6.6	4.6	6.0	6.6	23.3

TABLE VIII

ANALYSIS OF VARIANCE OF LEAF PROTEIN DATA

Source	Degrees of Freedom	Sum of Squares	Mean Square	F
Process	3	2465.78	821.93	191.51**
Moist cake vs Rest	1	0.59	0.59	<1 n.s.
Frozen vs Freeze-dried and Decolorized	1	1606.67	1606.67	374.36**
Freeze-dried vs Decolorized	1	858.52	858.52	200.04**
Amino Acids	7	7077.15	1011.02	235.57**
Process x Acids	21	897.64	42.74	9.95**
Acid x Moist cake vs Rest	7	195.04	27.86	6.49**
Acid x Frozen vs Freeze-dried and Decolorized	7	367.27	52.46	12.22**
Acid x Freeze-dried vs Decolorized	7	335.31	47.90	11.61**
Error	64	274.67	4.2917	
Total	95	10715.24		

** Significant at P = 0.01

highly significant. However, the analysis also indicated a significant interaction, which implied that the differences in responses to amino acids varied with the method of processing.

An examination of Figure 1 indicates the source of interaction. From this diagram, it is apparent that the decolorized leaf protein when supplemented with methionine, was no better than moist cake with a similar supplementation. The response curve of the lysine-supplemented decolorized leaf protein rose sharply when compared with other processes and the isoleucine-methionine combination resulted in a decline in growth when the decolorized sample was used as protein source. The isoleucine-lysine supplementation when applied to decolorized leaf protein had less effect than it did when added to the medium containing moist press-cake or frozen leaf protein. The addition of isoleucine to the frozen sample seemed to result in a greater increase over the unsupplemented when compared with the other processes.

Partitioning the process sum of squares in the analysis (Table VIII) showed significant differences between the freezing process and the two drying methods. In addition, there was a significant difference between the freeze-drying method and the decolorized in the favour of the decolorizing process. Because of the significant interaction between processes and amino acids, it was necessary to test for significance of amino acids with the various process contrasts (Table VIII). The highly significant results indicated that separate analyses of variance for the leaf protein processes should be computed.

Analysis of variance for the various processes showed a highly significant growth response to methionine supplementation (Tables IX -

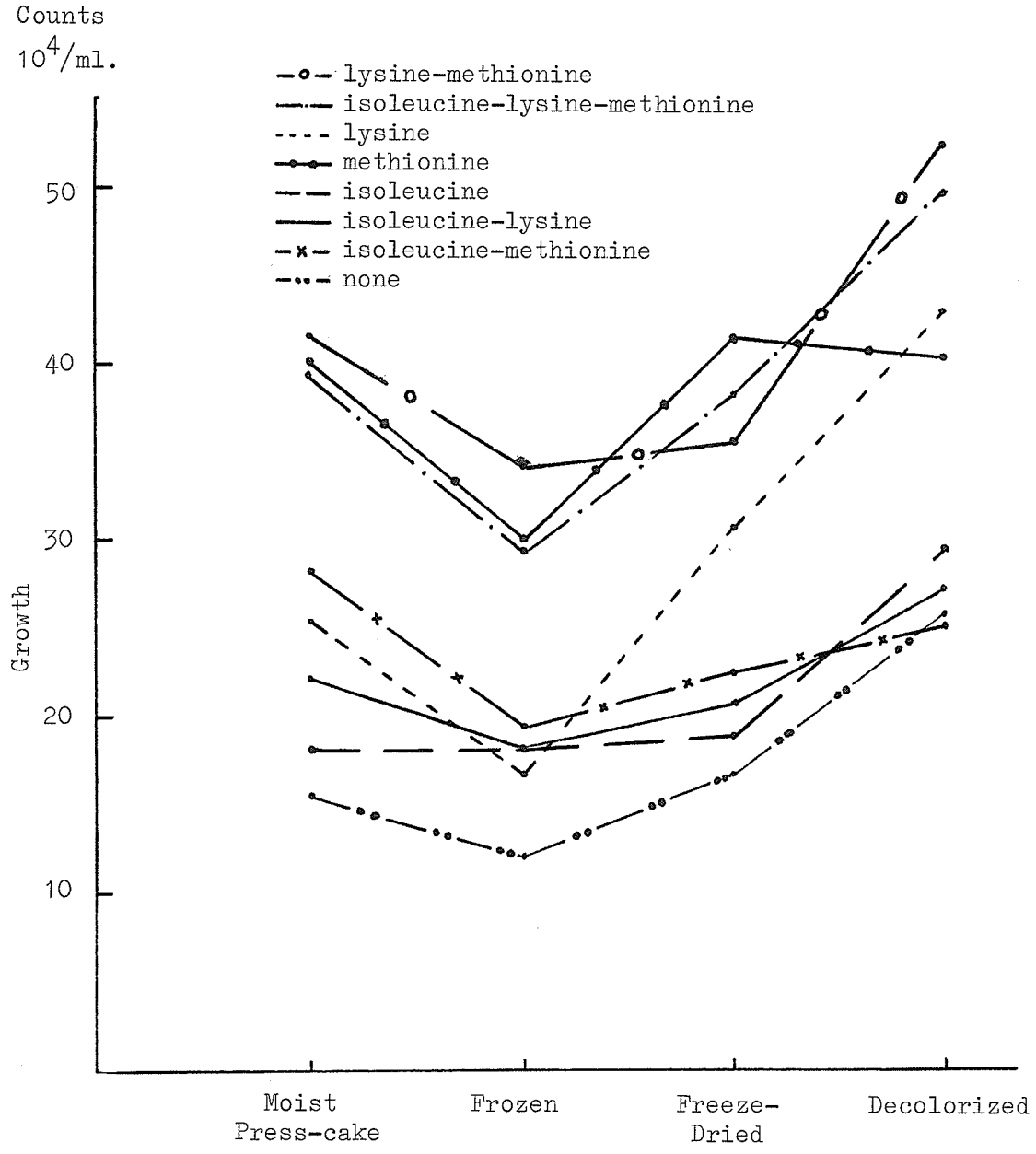


Figure 1. Effect of amino acid supplementation of leaf on growth of Tetrahymena pyriformis W.

XII). Henry and Ford (31) observed that the addition of DL-methionine to wheat leaf protein in amounts to equal that of casein, increased the biological value for rats from 75.0 to 81.5 and for tares leaf protein from 53.7 to 94.5. Figures 2 - 5 are plots of treatment means which indicate the growth response of the organism to additions of lysine and methionine when no isoleucine is added (a) and with isoleucine supplementation (b).

An examination of Figures 2 - 5 (a) shows that the increased growth with added methionine on moist cake and freeze-dried leaf protein, was much greater than that for the decolorized sample. This suggests that the moist cake would be as effective as the decolorized leaf protein for supplementing diets high in methionine, especially in countries where the green colour is not objectionable.

Although lysine in the leaf protein concentrates was lower than that of vitamin-free casein, the discrepancy was not great enough to warrant supplementation. Lysine, however, is the amino acid that causes most concern in the food industry because of its susceptibility to heat damage. Also, the Maillard reaction between its ϵ-NH_2 group and reducing sugars is well known. In order to avoid glucose-protein interaction, the glucose in the medium was autoclaved separately in all treatments.

Analysis of variance of the various leaf protein processes (Tables IX - XII) showed a significant growth response by the organism when crystalline L-lysine was added to the medium. Since the amount of lysine in the intact proteins was not remarkably low, this suggests that heat treatment during autoclaving may have rendered the lysine less available

TABLE IX

ANALYSIS OF VARIANCE OF MOIST PRESS-CAKE DATA

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F
Amino Acids	7	2250.69	321.52	68.84**
Met	1	1734.0	1734.0	371.30**
Lys	1	266.67	266.67	57.10**
Met x Lys	1	0.67	0.67	<1 n.s.
Ileu	1	80.67	80.67	17.27**
Met x Ileu	1	66.67	66.67	14.27**
Lys x Ileu	1	6.0	6.0	1.28 n.s.
Ileu x Met x Lys	1	96.0	96.0	20.55**
Error	16	74.68	4.67	
Total	23	2325.37		

** Significant at P = 0.01

TABLE X

ANALYSIS OF VARIANCE OF FROZEN LEAF PROTEIN DATA

Source	Degrees of Freedom	Sum of Squares	Mean Square	F
Amino Acids	7	1287.37	183.91	35.91**
Met	1	864.0	864.0	168.75**
Lys	1	130.67	130.67	25.52**
Met x Lys	1	32.67	32.67	6.38 n.s.
Ileu	1	24.0	24.0	4.68 n.s.
Met x Ileu	1	192.67	192.67	37.63**
Lys x Ileu	1	0.67	0.67	<1 n.s.
Ileu x Met x Lys	1	42.67	42.67	8.33 n.s.
Error	16	82.0	5.12	
Total	23	1369.37		

**Significant at P = 0.01

TABLE XI

ANALYSIS OF VARIANCE OF FREEZE-DRIED LEAF PROTEIN DATA

Source	Degrees of Freedom	Sum of Squares	Mean Square	F
Amino Acids	7	1920.31	274.33	83.38**
Met	1	950.04	950.04	288.76**
Lys	1	247.04	247.04	75.08**
Met x Lys	1	15.04	15.04	4.57 n.s.
Ileu	1	222.04	222.04	67.48**
Met x Ileu	1	26.04	26.04	7.91 n.s.
Lys x Ileu	1	35.04	35.04	10.65**
Ileu x Met x Lys	1	425.04	425.04	129.19**
Error	16	52.69	3.29	
Total	23	1973.0		

** Significant at P = 0.01

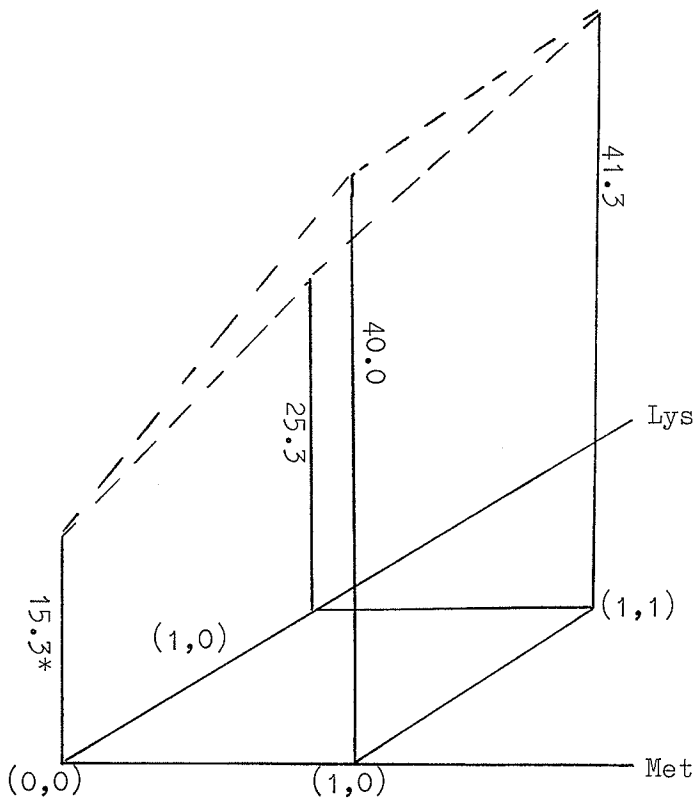
TABLE XII

ANALYSIS OF VARIANCE OF DECOLORIZED LEAF PROTEIN DATA

Source	Degrees of Freedom	Sum of Squares	Mean Square	F
Amino Acids	7	2516.50	359.50	88.11**
Met	1	661.50	661.50	162.13**
Lys	1	962.67	962.67	235.94**
Met x Lys	1	170.67	170.67	41.83**
Ileu	1	322.67	322.67	79.08**
Met x Ileu	1	10.67	10.67	2.61 n.s.
Lys x Ileu	1	20.17	20.17	4.94 n.s.
Ileu x Met x Lys	1	368.17	368.17	90.23**
Error	16	65.37	4.08	
Total	23	2581.87		

** Significant at P = 0.01

(a) All supplements except isoleucine



(b) Supplemented with isoleucine

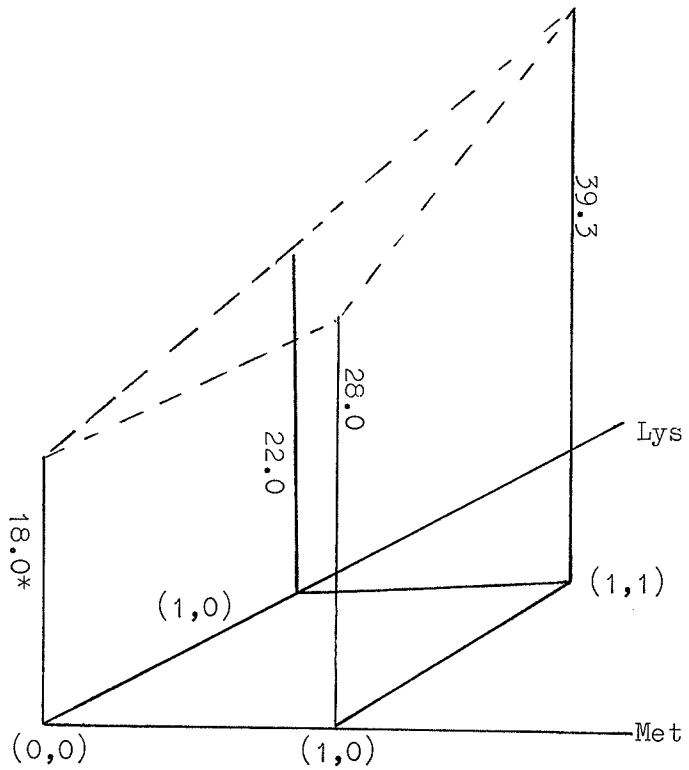


Figure 2. Plots of Treatment Means* for Moist Press-cake demonstrating interactions.

* Mean derived from Table VII.

(a) All supplements except isoleucine

(b) Supplemented with isoleucine

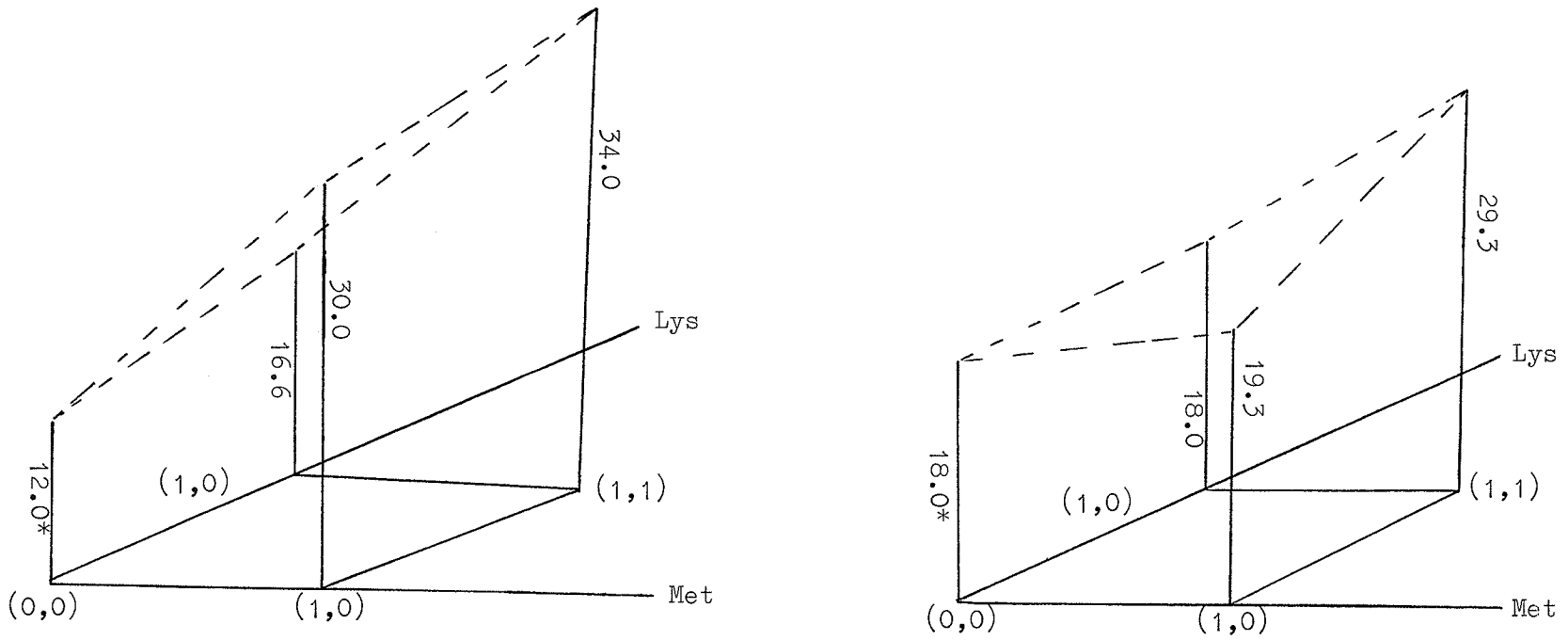


Figure 3. Plots of Treatment Means* for Frozen Leaf Protein demonstrating interactions.

*Means derived from Table VII

(a) All supplements except isoleucine

(b) Supplemented with isoleucine

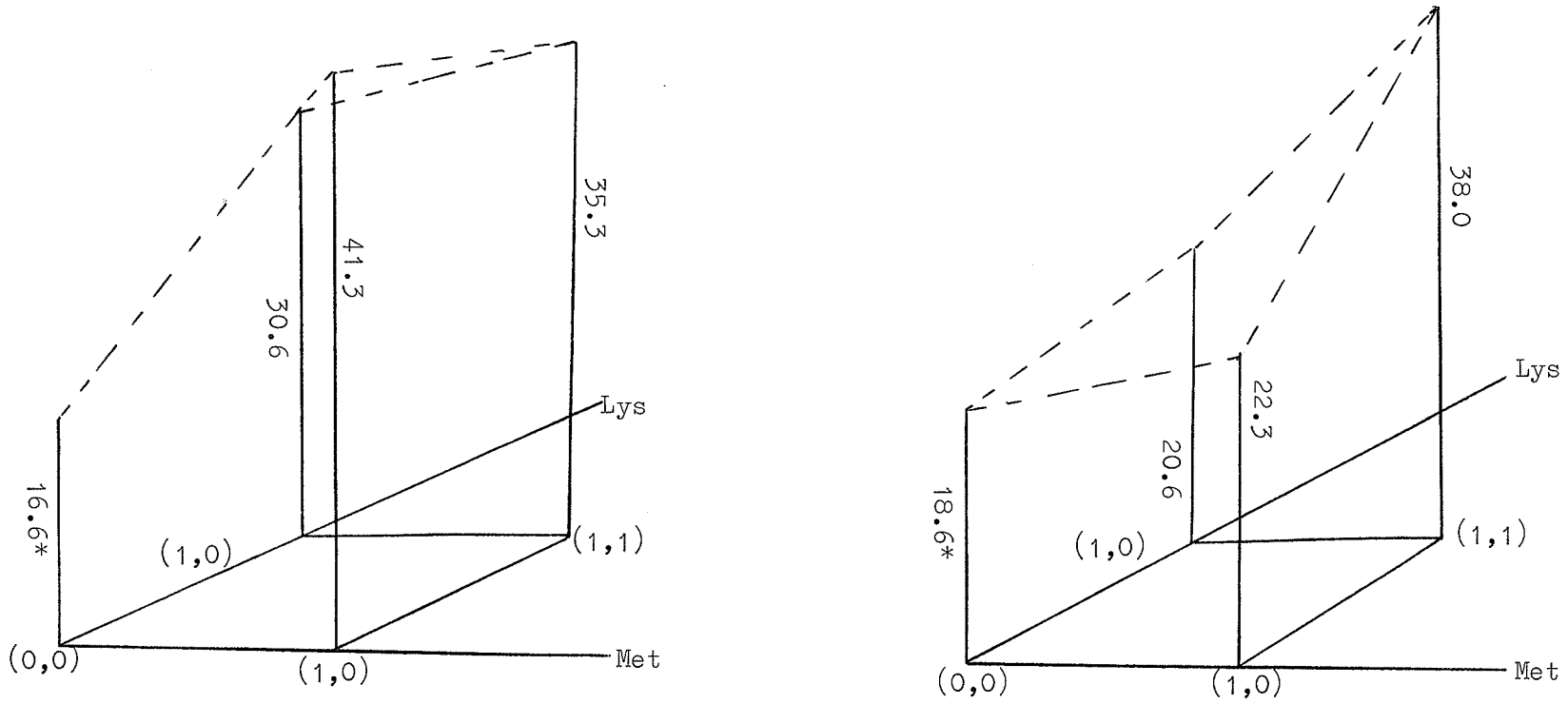
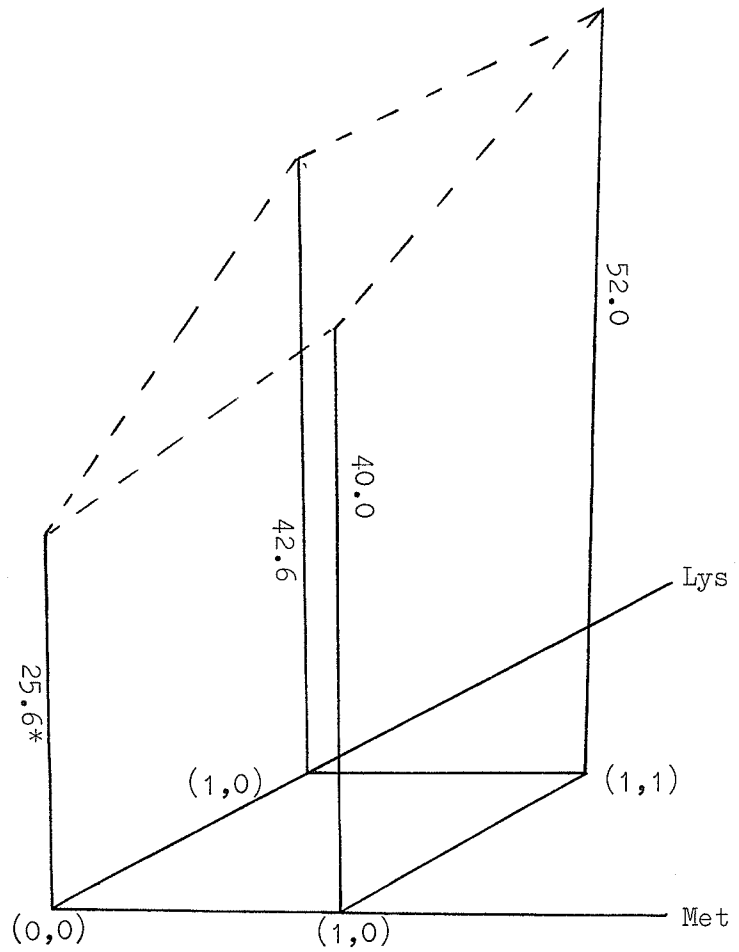


Figure 4. Plots of Treatment Means* for Freeze-dried Leaf Protein demonstrating interactions.

* Means derived from Table VII.

(a) All supplements except isoleucine



(b) Supplemented with isoleucine

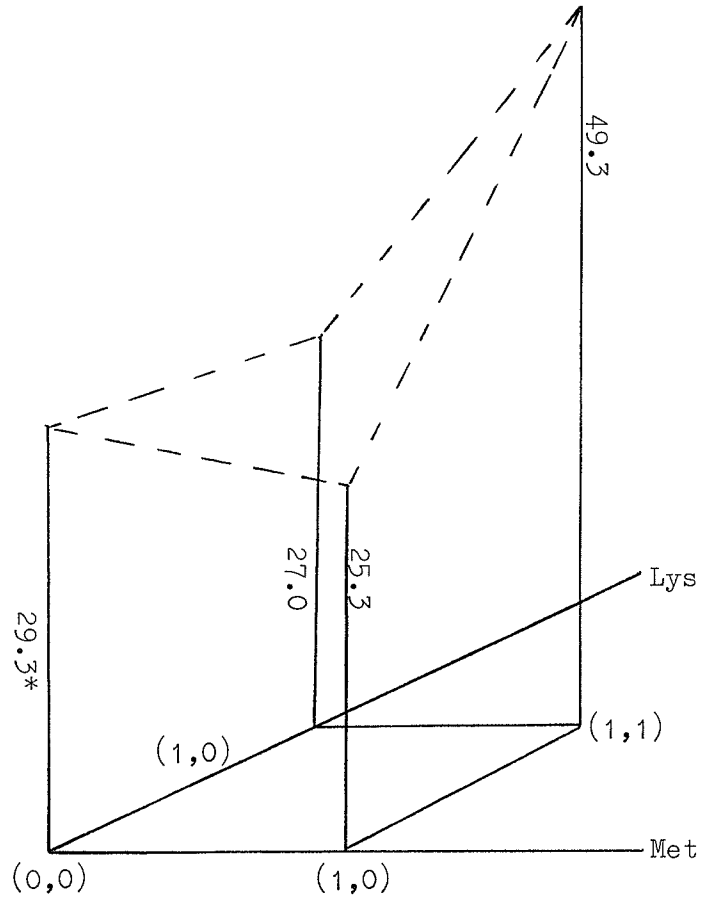


Figure 5. Plots of Treatment Means* for Decolorized Leaf Protein demonstrating interactions.

* Means derived from Table VII

due to its resistance to enzymic release from the proteins. An examination of Figures 2 - 5 (a) shows that of all the leaf proteins tested, the decolorized sample showed the greatest increase when L-lysine was added to the medium.

The addition of isoleucine in amounts to equal that of vitamin-free casein produced significantly negative results for all processes with the exception of the frozen sample (Tables IX - XII). This process resulted in a negative reaction that was not significant. Figure 1 helps to explain this result. The significant negative results for the other leaf protein processes indicated that the additional isoleucine contributed little or even inhibited the growth of the organism.

When isoleucine was supplied in the medium in combination with methionine or lysine, it appeared to cause depression in growth. Tables IX - XII show that the methionine-isoleucine interaction was significantly negative for the moist press-cake and frozen sample, and was not significant for the freeze-dried and decolorized leaf protein processes. Similarly, when isoleucine was added to the medium in combination with lysine, the interaction effect in all except the freeze-dried sample was not significant. (Tables IX - XII). These effects are clearly demonstrated in Figures 2 - 5 when comparisons are made with responses in the absence (a) and presence (b) of isoleucine. Isoleucine, then may not have been a limiting factor and when supplemented amounts were present, these proved to be inhibitory to the organism.

The three factor interaction caused by the addition of methionine-isoleucine-lysine, to the medium, was highly significant for all treat-

ments except the frozen leaf protein (Tables IX - XII). Although growth on this triple supplementation was generally better than some of the single supplementations, Figures 2 - 5 (b) (1,1) show that isoleucine was again acting to reduce the effect of the methionine-lysine supplementation seen at (1,1) Figures 2 - 5 (a). An understanding of the mechanism underlying the inhibitory effect of isoleucine when combined with other amino acids requires further experimentation.

The analyses of variance (Tables IX - XII) showed that the methionine-lysine interaction was not siginficant for any treatment with the exception of the decolorized leaf protein. An examination of Figures 2 - 5 (a) shows that this combination (1,1) resulted in the best growth for all treatments with the exception of the freeze-dried leaf protein. The non-significant interaction for the three green processes indicated that both methionine and lysine when added to the leaf protein treatments were acting independently to cause good growth as evidenced by the positive slopes in Figures 2 - 5 (a). The significant interaction for the decolorized sample implies that the addition of methionine further intensified the growth-promoting effect of lysine supplementation. Figure 5 (a) shows the excellent response to the methionine-lysine combination (1,1) when the organism was grown on decolorized leaf protein.

Comparisons of the protozoal method with conventional methods

The value of any particular procedure can only be judged by the extent to which it gives results that correlate with those tested by valid animal or human biological assays.

In an effort to express in a more meaningful way the growth of micro-

organisms on various foodstuffs, Ford (27) used relative nutritive values (RNV's). This is an expression of the nutritive value of a food tested under similar conditions as casein, which is arbitrarily assigned a value of 100.

Fernell and Rosen (26) have determined the relative nutritive values (RNV's) of a variety of proteins using Tetrahymena pyriformis W and compared the results with literature values for chemical score, net protein utilization, and protein efficiency ratios in the rat. The values obtained for Tetrahymena agreed with those determined in the rat when the values were expressed relative to casein which was assigned the value of 100. Egg albumin with a chemical score of 113 had an RNV of 121 with Tetrahymena pyriformis W and on NPU of 122. Soybean meal with a chemical score of 87 gave an RNV of 83 and an NPU of 106 in the rat. Groundnut meal with a chemical score of 51 gave an RNV of 45. The RNV was found to vary with the nitrogen range selected for comparison, with low quality proteins giving high RNV's at higher nitrogen levels. It is therefore important to use a nitrogen level less than 1 mg. N/ml. in order to obtain a true assessment of protein quality.

The relative nutritive values for the various leaf protein treatments tested in this study are given in Table XIII. The decolorized leaf protein supplemented with lysine plus methionine gave an RNV of 94.5 and that of the sample supplemented with lysine plus isoleucine and methionine had a relative nutritive value for the organism of 89.6. For the moist cake, the highest RNV of 75.1 was obtained for the methionine-lysine supplementation.

TABLE XIII

RELATIVE NUTRITIVE VALUES (RNV's) FOR TREATMENTS OF LEAF PROTEIN (CASEIN = 100)

Amino Acid Supplementation	Moist Press-Cake	Frozen Leaf Protein	Freeze-Dried Leaf Protein	Decolorized Leaf Protein	Oven-Dried Leaf Protein
None	27.8	21.8	30.1	46.5	3.6
Met	72.7	54.5	75.0	72.7	5.4
Ileu	32.7	32.7	33.8	53.2	15.6
Lys	46.0	30.1	55.7	77.4	12.0
Ileu + Met	50.9	35.0	40.5	46.0	8.3
Ileu + Lys	40.2	32.7	37.4	49.1	10.9
Lys + Met	75.1	61.8	64.1	94.5	12.0
Ileu + Lys + Met	71.4	53.2	69.0	89.6	42.3

The decolorized leaf protein with methionine-lysine supplementation was a better protein source for Tetrahymena pyriformis than soybean meal and groundnut meal tested by Fernell and Rosen (26), but not as good as egg albumin.

The hypothetical values that can be applied for NPU of the test leaf proteins give a fairly good indication of the value of these treatments if fed to rats. Henry and Ford (31) found that wheat leaf proteins supplemented with methionine gave an NPU for rats of 70.6 and tares leaf protein with similar supplementation had an NPU for the same rats of 78.5. The true digestibilities of these leaf samples were 85% and 83% respectively.

Boyne and co-workers (9) considering the results of meat meals observed that nutritive values with Tetrahymena pyriformis showed a correlation with available lysine values and gross protein values (GPV) determined by chick feeding tests. The GPV for chicks measures the value of a protein source as a supplement to cereal diets. Results for fish meals however, showed no correlation with available lysine values, perhaps because lysine was not the limiting amino acid for Tetrahymena pyriformis in these meals. Ford (27) using Steptococcus zymogenes to measure RNV's of a variety of proteins demonstrated a close correlation with the GPV's reported by Boyne (9).

It was of interest to determine whether the RNV of the better leaf proteins in this study correlated with GPV's for leaf proteins reported in the literature. Duckworth and Woodham (22) have reported mean GPV's for five types of leaf protein - barley, rye, tares, kale and mixed

grasses to be 79 for chicks. The RNV's of the leaf proteins seem to correlate fairly well with the GPV's reported.

Very little is known about the quantitative amino acid requirements of Tetrahymena pyriformis W or the factors that control the relative ingestion of particulate and dissolved nutrients when both are supplied in the medium. Nevertheless the inferiority of the leaf proteins when compared with vitamin-free casein is testimony of the ability of the organism to discriminate between proteins of different qualities.

The microbiological method described in this study holds promise of a rapid, inexpensive protein screening method. The response of the organism to amino acid supplementation, illustrated by the effect of addition of methionine and lysine to the leaf concentrates increases confidence in the method.

Perhaps a drawback to the Tetrahymena method is the apparent inhibitory effect of free fatty acids as demonstrated by the better growth on decolorized leaf protein. If further studies confirm this hypothesis, then preliminary defatting of meals that are to be assayed by this method would be required in order that growth of the organism may truly reflect biological availability of protein. The chlorophyll and acid breakdown products present in the leaf protein samples may also have been responsible for a degree of retardation of growth of the organism. Thus the need for further experimentation of acetone-soluble materials is demonstrated.

SUMMARY AND CONCLUSIONS

A protozoal method for evaluation of protein quality was tested. The organism used was Tetrahymena pyriformis W and the test material was leaf protein concentrates processed by various methods. Vitamin-free casein, the reference standard, was provided at a level of 0.5 mg N/ml. as were all leaf protein samples.

Chenopodium quinoa, grown under controlled greenhouse conditions, was used as the leaf protein source. The concentrates were prepared according to the method of Morrison and Pirie (50), and portions of the moist press-cake were quick frozen, freeze-dried, decolorized with acetone, oven-dried at 65°C for four hours or refrigerated.

The assay procedure for determination of the growth response of the organism was essentially that of Stott, Smith and Rosen (66). Direct microscopic count was chosen in preference to the conventional turbidity and colorimetric procedures because of the insoluble and coloured materials in the medium.

Amino acid composition of the leaf concentrates compared favourably with vitamin-free casein except for methionine, which was demonstrated to be the first limiting factor.

When compared with casein, the unsupplemented leaf protein preparations with the exception of the decolorized sample resulted in poor growth. The improved growth response of the organism when fed the decolorized leaf protein suggests that the acetone soluble material present in the leaf may have been inhibitory to the organism. The oven-dried leaf protein, because of its insolubility, proved to be a most inferior

source of nitrogen for the organism.

Addition of crystalline DL-methionine and L-Lysine in amounts to equal that of casein, resulted in improved growth for all treatments. Analysis of variance of the supplementation data showed that the main effects, processes and amino acids, were highly significant. There was also a significant interaction, which implied that the differences in responses to amino acids varied with the method of processing. L-isoleucine added singly or in combination with DL-methionine or L-lysine generally depressed growth. The methionine-lysine supplementation evoked the best growth response from Tetrahymena pyriformis W. Of all the processes, the decolorized was by far the most superior.

Relative nutritive values, (i.e. relative to casein taken as 100) obtained for the better leaf protein concentrates, seemed to correlate fairly well with the GPV's of leaf proteins tested by other workers (22).

The microbiological method described in this study demonstrated its potentialities as a rapid screening method. The organism was able to differentiate between good and inferior quality protein and responded to amino acid supplementation, which in view of the widening interest in the utilization of vegetable protein sources, extends the potentialities of the assay procedure. Perhaps one drawback to the use of Tetrahymena pyriformis W lies in the apparent inhibitory effect of free fatty acids which would necessitate the defatting of meals for routine assays.

The leaf protein concentrates tested in this study, proved in some cases to be superior to soya bean and groundnut meal, but poorer than

casein. Thus, when combined with other foods high in methionine, leaf protein could definitely enhance their value and provide an economical supplement to the cereal-based diets deficient in good quality protein.

REFERENCES

1. Akeson, W. R. and Stahmann, M. A. 1964. A pepsin pancreatin digest index of protein quality evaluation. J. Nutr. 83, 257.

2. Allison, J. B. 1955. Biological evaluation of protein. Physiol. Rev. 35, 664.

3. Almquist, H. J., Stokstad, E. L. R., and Halbrook, E. R. 1935. Supplementary values of animal protein concentrates in chick rations. J. Nutr. 10, 193.

4. Anderson, M. E. and Williams, H. H. 1951. Microbiological evaluation of protein quality. 1. A colorimetric method for the determination of the growth of Tetrahymena geleii W in protein suspensions J. Nutr. 44, 335.

5. Association of Agricultural Chemists. 1965. Official methods of analysis. 10th Edition. A.O.A.C., Washington, D.C.

6. Barber, R. S., Braude, R., and Mitchell, K. G. 1959. Leaf protein in rations of growing pigs. Proc. Nutr. Soc. 18, iii.

7. Bender, A. E. and Doell, D. H. 1957. Biological evaluation of proteins. A new aspect. Brit. J. Nutr. 11, 140.

8. Block, P. J. and Mitchell, H. H. 1946. The correlation of the amino acid composition of proteins with their nutritive value. Nutr. Abstr. Rev. 16, 249.

9. Boyne, A. W., Carpenter, K. J. and Woodham, A. A. 1961. Progress report on an assessment of laboratory procedures suggested as indicators of protein quality in feeding stuffs. J. Sci. Food Agr. 12, 832.

10. Bressani, R. 1960. Supplementation of cereal proteins with amino acids. III. Effect of amino acid supplementation of wheat flour as measured by nitrogen retention of young children. J. Nutr. 70, 176.

11. Bressani, R. 1963. Supplementation of cereal proteins with amino acids. V. Effect of supplementing lime treated corn with different levels of lysine, tryptophan and isoleucine on the nitrogen retention of young children. J. Nutr. 80, 80.

12. Bressani, R., Wilson, D., Behar, M., Chung, M., and Scrimshaw, N. S. 1963. Supplementation of cereal proteins with amino acids. IV. Lysine supplementation of wheat flour fed to young children at different levels of protein intake in the presence and absence of other amino acids. J. Nutr. 79, 333.

13. Bunyan, J. and Price, S. A. 1960. Studies on protein concentrates for animal feeding. J. Sci. Food Agr. 11, 25.

14. Byers, M., Green, S. H., and Pirie, N. W. 1965. The presentation of leaf protein on the table. II. Nutrition 19, 63.

15. Cannon, P. R., Humphreys, E. M., Wissler, R. W., and Frazier, E. L. 1944. The effects of feeding possible blood substitutes on serum protein regeneration and weight recovery in the hypoproteinemic rat. J. Clin. Invest. 23, 601.

16. Carpenter, K. J. 1960. The estimation of 'available' lysine in animal-protein foods. Biochem. J. 77, 604.

17. Carpenter, K. J. and Ellinger, G. M. 1955. The estimation of 'available' lysine in protein concentrates. Biochem. J. 61, xi.

18. Carpenter, K. J., Ellinger, G. M., and Shrimpton, D. H. 1955. The evaluation of whaling by-products as feeding stuffs. J. Sci. Food Agr. 6, 296.

19. Chibnall, A. C., Rees, M. W., and Lugg, J. W. H. 1963. Amino acid composition of leaf protein. J. Sci. Food Agr. 14, 234.

20. Corliss, J. O. 1953. Comparative studies on holotrichous ciliates in the Colpidium-Glaucoma-Leucophrys-Tetrahymena group. II. Morphology, life cycles and systematic status of strains in pure culture. Parasitology 43, 49.

21. Davys, M. N. G. and Pirie, N. W. 1963. Batch production of protein from leaves. J. Agr. Eng. Res. 8, 70.

22. Duckworth, J. and Woodham, A. A. 1961. Leaf protein concentrates. I. Effect of source of raw material and method of drying on protein value for chicks and rats. J. Sci. Food Agr. 12, 5.

23. Duckworth, J., Hepburn, W. R., and Woodham, A. A. 1961. Leaf protein concentrates. II. The value of a commercially dried product for newly weaned pigs. J. Sci. Food Agr. 12, 16.

24. Dunn, M. S. and Rockland, L. B. 1947. Biological value of proteins determined with Tetrahymena geleii H. Proc. Soc. Exptl. Biol. Med. 64, 377.

25. Evans, R. J. and St. John, J. L. 1945. Estimation of the relative nutritive value of vegetable proteins by two chemical methods. J. Nutr. 30, 209.

26. Fernell, W. R. and Rosen, G. D. 1956. Microbiological evaluation of protein quality with Tetrahymena pyriformis W. I. Characteristics of growth of the organism and determination of relative nutritive values of intact proteins. Brit. J. Nutr. 10, 143.

27. Ford, J. E. 1960. A microbiological method for assessing the nutritional value of proteins. Brit. J. Nutr. 14, 485.

28. Ford, J. E. 1962. A microbiological method for assessing the nutritional value of proteins. II. The measurement of 'available' methionine, leucine, isoleucine, arginine, histidine, tryptophan and valine. Brit. J. Nutr. 16, 409.

29. Gerloff, E. D., Lima, I. H., and Stahmann, M. A. 1965. Amino acid composition of leaf protein concentrates. J. Agr. Food. Chem. 13, 139.

30. Halevy, S. and Grossowicz, N. 1953. A microbiological approach to nutritional evaluation. Proc. Soc. Exptl. Biol. Med. 82, 567.

31. Henry, K. M. and Ford, J. E. 1965. The nutritive value of leaf protein concentrates determined in biological tests with rats and by microbiological methods. J. Sci. Food Agr. 16, 425.

32. Horn, M. J., Blum, A. E., Womack, M., and Gersdoff, C. E. F. 1952. Nutritional evaluation of food proteins by measuring availability of amino acids to microorganisms. I. Cottonseed protein. J. Nutr. 48, 231.

33. Horn, M. J., Blum, A. E., and Womack, M. 1954. Availability of amino acids to microorganisms. J. Nutr. 52, 375.

34. Howe, E. E., Gilfillan, E. W., and Allison, J. B. 1960. Efficacy of the FAO amino acid reference standard for growth of the weanling rat. J. Nutr. 70, 385.

35. Jansen, G. R. 1969. Total protein value of protein - and amino acid - supplemented bread. Am. J. Clin. Nutr. 22, 38.

36. Kidder, G. W. and Dewey, V. C. 1951. In "Biochemistry and Physiology of the Protozoa," ed. Lwoff, A. pp. 323. Academic Press, New York.

37. Kuhnau, J. 1949. The biochemistry of natural proteins. Angew. Chem. 61, 357.

38. Leverton, R. M. and Steel, D. 1962. Nitrogen balances of young women fed the FAO reference pattern of amino acids and the oat pattern. J. Nutr. 78, 10.

39. Lima, I. H., Richardson, T., Stahmann, M. A. 1965. Fatty acids in some leaf protein concentrates. J. Agr. Food Chem. 13, 143.

40. Mauron, J., Mottu, F., Bujard, E., and Egli, R. H. 1955. The availability of lysine, methionine and tryptophan in condensed milk and milk powder. In vitro digestion studies. Arch. Biochem Biophys. 59, 433.

41. Mauron, J., Mottu, F. 1958. Relationship between in vitro lysine availability and in vivo protein evaluation in milk powders. Arch. Biochem. Biophys. 77, 312.

42. Melnick, D., Oser, L. B., and Weiss, S. 1946. Rate of enzymic digestion of proteins as a factor in nutrition. Science. 103, 326.

43. Mertz, E. T., Rennert, S. S., and Cole, E. W. 1955. A bacterial method for determining protein digestibility. J. Nutr. 56, 437.

44. Miller, D. S. and Bender, A. E. 1955. The determination of the net utilization of proteins by a shortened method. Brit. J. Nutr. 9, 382.

45. Miller, D. S. and Payne, P. R. 1961. Problems in the prediction of protein values of diets. The influence of protein concentration. Brit. J. Nutr. 15, 11.

46. Mitchell, H. H. 1923. A method of determining the biological value of proteins. J. Biol. Chem. 58, 873.

47. Mitchell, H. H. 1954. In "Symposium on Methods for the Evaluation of Nutritional Adequacy and Status," eds. Spector, H., Peterson, M. S., Friedemann, T. E. pp. 13. National Research Council, Washington, D. C.

48. Mitchell, H. H. and Block, R. J. 1946. Some relationships between the amino acid contents of proteins and their nutritive value for the rat. J. Biol. Chem. 163, 599.

49. Morrison, J. E. and Pirie, N. W. 1960. The presentation of leaf protein on the table. Nutrition. 14, 7.

50. Morrison, J. E. and Pirie, N. W. 1961. The large scale production of protein from leaf extracts. J. Sci. Food Agr. 12, 1.

51. National Academy of Sciences - National Research Council. 1963. Evaluation of protein quality. Report of an International Conference. Committee on Protein Malnutrition. Food and Nut. Board. Publication 1100. Washington, D.C.

52. Osborne, T. B., Mendel, L. B., and Ferry, E. L. 1919. A method of expressing numerically the growth promoting value of proteins. J. Biol. Chem. 37, 223.

53. Oser, B. L. 1951. Method for integrating essential amino acid content in the nutritional evaluation of protein. J. Am. Dietet. Assoc. 27, 396.

54. Pilcher, H. L. and Williams, H. H. 1954. Microbiological evaluation of protein quality. J. Nutr. 53, 589.

55. Pirie, N. W. 1952. Large scale production of edible protein from fresh leaves. Ann. Rept. Rothamsted. Expt. Sta. for 1952. pp. 173. Harpenden, Herts.

56. Pirie, N. W. 1959. Leaf protein as human food. Lancet II, 961.

57. Pirie, N. W. 1968. The use of plant protein concentrates as human food. Chem and Ind. 26, 864.

58. Platt, B. S. and Miller, D. S. 1959. The net dietary protein value (N-D-p.V) of mixtures of foods - its definition, determination and application. Proc. Nutr. Soc. 18, vii.

59. Pleshkov, B. P. and Fowden, L. 1959. Amino acid composition of the proteins of barley leaves in relation to the mineral nutrition and age of plants. Nature. 183, 1445.

60. Rockland, L. B. and Dunn, M. S. 1949. Determination of the biological value of proteins with Tetrahymena geleii H. Food Technol. 3, 289.

61. Rosen, G. D. and Fernell, W. R. 1956. Microbiological evaluation of protein quality with Tetrahymena pyriformis W. II. Relative nutritive value of proteins in foodstuffs. Brit. J. Nutr. 10, 156:

62. Sheffner, A. L., Eckfeldt, G. A. and Spector, H. 1956. The pepsin digest-residue (PDR) amino acid index of net protein utilization. J. Nutr. 60, 105.

63. Sheffner, A. L., Adachi, R. and Spector, H. 1956. Measurement of the net utilization of heat processed proteins by means of the pepsin-digest-residue (PDR) amino acid index. J. Nutr. 60, 507.

64. Steel, R. D. and Torrie, J. H. 1960. "Principles and Procedures of Statistics", McGraw-Hill Book Company, Inc., New York.

65. Stott, J. A. and Smith, H. 1960. Microbiological assay of protein quality with Tetrahymena pyriformis W. IV. Measurement of available lysine, methionine, arginine and histidine. Brit. J. Nutr. 20, 663.

66. Stott, J. A., Smith, H. and Rosen, G. D. 1963. Microbiological evaluation of protein quality with Tetrahymena pyriformis W. III. A simplified assay procedure. Brit. J. Nutr. 17, 227.

67. Subba Rao, M. S., Singh, N., Prasannappa, G. 1967. Preservation of wet leaf protein concentrates. J. Sci. Food Agr. 18, 295.

68. Teeri, A. E., Virchow, W. and Loughlin, M. E. 1956. A microbiological method for the nutritional evaluation of proteins. J. Nutr. 59, 587.

69. Teunisson, D. J. 1961. Microbiological assay of intact proteins using Tetrahymena pyriformis W. I. Surveys of protein concentrates. Anal. Biochem. 2, 405.

70. Viswanatha, T. and Liener, I. E. 1955. Utilization of native and denatured proteins by Tetrahymena pyriformis W. Arch. Biochem. Biophys. 56, 222.

71. Waterlow, J. C. 1962. The absorption and retention of nitrogen from leaf protein by infants recovering from malnutrition. Brit. J. Nutr. 16, 531.

72. Waterworth, D. G. 1964. The nutritive quality and available amino acid composition of some animal protein concentrates. Brit. J. Nutr. 18, 503.

73. World Health Organization. 1965. Protein requirements. Report of a joint FAO/WHO expert group. Technical Report Series, 301. Geneva.

APPENDIX

APPENDIX

ORIGINAL LEAF PROTEIN DATA

Amino Acids	Triplicate Treatment Values														
	Moist Press-Cake			Frozen Leaf Protein			Freeze-Dried Leaf Protein			Decolorized Leaf Protein			Oven-Dried Leaf Protein		
None	16	14	16	14	10	12	16	16	18	28	25	24	2	2	2
Methionine	40	38	42	30	32	28	42	42	40	40	42	38	3	4	3
Isoleucine	18	16	20	16	20	18	20	18	18	30	28	30	10	6	10
Lysine	26	26	24	17	19	14	30	32	30	44	40	44	4	8	8
Isoleucine & Methionine	30	26	28	20	20	18	22	24	21	26	28	22	5	4	5
Isoleucine & Lysine	24	22	20	16	18	20	20	24	18	28	25	28	7	4	7
Lysine & Methionine	42	38	44	34	30	38	34	38	34	52	50	54	4	10	6
Isoleucine & Lysine & Methionine	40	36	42	30	28	30	36	40	38	50	50	48	26	20	24