Assessment of the Nutritional Value of Novel Proteins

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Abstract

Methods of determining the nutritive value of novel proteins or derived food products such as proteins from fish, algae, leaf or microbiological sources, are reviewed. The use of physical (solubility) or chemical (amino acid analysis) methods, feeding tests in rats and other animals for the determination of biological value, digestibility and protein efficiency ratios, rate of enzymatic hydrolysis or retention of enzymatic activity are among the procedures surveyed. The safety of new food sources must always be a first consideration.

Novel sources of food protein may be derived not only from oil seeds but also from many other potential sources such as fish, algae and microorganisms. Much attention is being directed to single cell proteins, a euphemism for yeasts or bacteria grown on methane from natural gas, or on petroleum hydrocarbons, molasses, wood pulp or other organic substrates. These will in time come to be used as raw materials for the production of food proteins. In general, the procedures now used for the assessment of food safety are applicable to these novel proteins.

The nutritional properties of new forms of protein are not the only criteria of their value. Obviously we must consider first the safety of these proteins relative to the sources of the raw materials and the conditions under which they are processed. With microbial proteins, the viability and nonpathogenicity of the organisms and their capacity to mutate into what might be pathogenic forms must be considered. To use the old cliche, no food is a food until it can be eaten. Acceptability takes into account not only taste, odor and texture, but also religious, esthetic and cultural preferences.

New proteins also must have the physical properties which suit them to technological application. They must be made economically available to the people who need them. It does no good to produce fish protein concentrate if it does not get to those it would help. Fresh fish, for example, available along the seaboard in many countries could be a more valuable resource if proper refrigeration for its transport deep into the land areas were available.

Before discussing the nutritional evaluation of these novel proteins, let us explore the various factors involved in safety evaluation since they bear considerably on the nutritional aspect. Chemical examination is usually the starting point. Analyses for the proximate composition and nitrogen partition are made, and these include amino acid composition as well as identification of nonprotein nitrogenous components. The presence of contaminants which may be naturally occurring toxic agents of plant origin or pesticide residues should be considered. Examples of toxins of plant origin include gossypol, lathyrogens, goitrogens, carcinogens, hemagglutinins, cyanogenetic glycosides, estrogens and trypsin inhibitors. These have to be considered from the very beginning because it is easy to confuse effects due to a toxic agent with those induced by proteins of inferior nutritional value.

Processing may actually improve the nutritional value of such proteins but under some conditions may diminish it. Proper heat processing can destroy antitryptic factors in the leguminous proteins and enhance biological values. Nutritional value can be reduced by excessive heat, especially in the presence of carbohydrates, which cause binding of amino acids (particularly lysine and methionine).

Some of the procedures used to assess the effect of processing or storage on nutritional value are: (a) Physical: solubility in H_2O, 3% NaCl, 5% KCl. (b) Chemical: total amino acid spectrum; available lysine. (c) Biochemical: rate and degree of enzyme hydrolysis, pepsin, pepsin + trypsin; residual enzyme (soy urease or antitrypsin). (d) Biological: digestibility (absorption); biological value (retention); NPU, PER. Chemical analyses include the determination of the amino acid spectrum of the acid hydrolysate, except for tryptophan for which the alkaline hydrolyzate must be employed. Biochemical examinations which simulate the effect of gastrointestinal digestive enzymes on the protein are conducted to determine the rate and degree of hydrolysis. The efficacy of heat processing is sometimes gauged by assaying, not for the substance to be destroyed, but for a more readily determined concomitant enzyme. For example, urease activity in soybean meal parallels the antitryptic activity. Final evaluation consists of biological tests for digestibility and utilization which will be discussed later.

Essential amino acids are defined principally in terms of ability to meet requirements for growth and maintenance. They are not synthesized by the body at a rate sufficient to permit optimal growth. An almost infinite array of proteins are synthesized to produce the organs and tissues of the body and vary from muscle and bone matrix to hair and fingernails. In addition to tissue proteins, special functional proteins are synthesized in vivo, such as hemoglobin, enzymes, hormones, antibodies, milk proteins and so on ad infinitum. Proteins do indeed play a protean role.

The essential amino acids (EAA) were originally categorized on the basis of rat experiments and later by studies of the requirements of young human adults. Eight amino acids listed in the first column of Table I are usually regarded as the "essentials." Cystine is included with methionine because of its sparing action when both are present in the diet, and similarly tyrosine is included with phenylalanine because it also has some value in replacement of

TABLE I

Essential Amino Acids

Amino acid	Rose (1957)	FAO (1957)	EAA Index: Oser (1951)	EAA Index: Mitchell (1954)
Lysine	x	x	x	x
Tryptophan	x	x	x	x
Isoleucine	x	x	x	x
Valine	x	x	x	x
Arginine			x	
Methionine (Cystine)	x	x	x	x
Threonine	x	x	x	x
Leucine	x	x	x	x
Phenylalanine (Tyrosine)	x	x	x	x
Histidine			x	x

Presented at AOCS-AACC Short Course on Oilseed Proteins: Chemistry, Technology and Economics, French Lick, Indiana, July 1969.

TABLE II

The FAO Pattern and the Proteins of Egg, Human Milk, and Cow's Milk Compared With Amino Acid Requirements of Human Beings[a] (3)

Amino acid	FAO pattern	Egg	Human milk	Cow's milk	Pattern of amino acid requirements			
					Infant	Child	Adult	
							Female	Male
Arginine	....	6.6	4.1	3.7	....	....	....	....
Histidine	....	2.4	2.2	2.7	2.4	....	....	....
Lysine	4.2	6.6	6.6	7.9	7.7	10.7	5.1	5.1
Leucine	4.8	8.8	9.1	10.0	10.9	8.0	6.1	7.0
Isoleucine	4.2	6.6	5.5	6.5	6.6	5.3	4.6	4.5
Methionine	2.2	3.1	2.3	2.5	4.8	....	3.5	1.3
Cystine	....	2.3	2.0	0.9	....	....	2.1	5.1
Total S-acids	4.2	5.4	4.3	3.4	6.2[b]	4.8[b]	5.6	6.4
Phenylalanine	2.8	5.8	4.4	4.9	6.6	4.8	2.2	1.9
Tyrosine	2.8	5.0	5.5	5.1	....	....	9.1	7.0
Total aromatic acids	5.6	10.8	9.9	10.0	....	....	11.3	8.9
Threonine	2.8	5.0	4.5	4.7	4.4	6.1	3.0	3.2
Tryptophan	1.4	1.7	1.6	1.4	1.6	1.6	1.6	1.6
Valine	4.2	7.4	6.3	7.0	6.7	5.9	6.6	5.1

[a] Expressed as g/16 g N.
[b] Methionine requirement in the absence of cystine, from Reference 3.

phenylalanine. The Food and Agricultural Organization (FAO) of the United Nations established a reference pattern of EAA requirements for man which was based on these eight essential amino acids. To predict nutritive value from the levels of the EAAs in proteins in 1951, these eight plus arginine and histidine were used since earlier animal experiments suggested that they could not be synthesized at a rate sufficient for the young adult male (1). There are many functions of amino acids that have not yet been fully assessed in relation to amino acid requirements, e.g., spermatogenesis, organogenesis and various endocrinological functions including lactation. From these early considerations the author developed an EAA index (1) which Mitchell (2) later modified by dropping arginine from the list of "essentials" and including tyrosine with phenylalanine.

EAA indexes provide an especially useful shortcut approach for predicting the nutritional value of proteins and for estimating the mutual supplementary effect of different proteins on each other. The EAA content of proteins is evaluated in comparison with either the human amino acid requirements or a reference protein. The former is rather limiting because Rose's figures and the FAO pattern are based largely on studies in normal adult males. We are concerned with the requirements of both sexes at all ages, and in various stages of health, disease or convalescence. A common practice has been to base comparisons on the EAA composition of an ideal protein. For example, the ideal protein for the infant would be that of human milk. Whole egg protein has become widely used as the complete reference protein because amino acid supplementation does not enhance its nutritive value. Table II, from a recent monograph of the NAS-NRC (3), shows the FAO pattern, and the EAA composition of the protein of egg, human and cow's milk, in relation to requirements at different ages. The similarity between human milk protein and that of whole egg is noteworthy. Table IV illustrates the composition of several cereal proteins in relation to whole egg protein.

Mitchell and Block (4) proposed a chemical method for rating protein, called the Chemical Score. This was based on the single amino acid in greatest deficit (Table III) compared with its content in whole egg protein. For example, the score for whole wheat is 39, the first limiting amino acid being lysine. The estimation of the availability of one amino acid, lysine, albeit by a chemical method, has proved especially useful as a quality control procedure for heat processed proteins. When lysine is bound intramolecularly or by carbohydrate it becomes unavailable and this results in a diminution in biological value of the protein. Measuring the combined biological value of eight chemical entities as different as the essential amino acids is like running a single assay for all of the vitamins in a food. It seems illogical to take just one limiting amino acid as the basis for rating a protein for its nutritional value. The probability theory would suggest that the rate of synthesis of a protein is a function not of any single amino acid level nor of the sum of the essential amino acid content, but rather of their availability. The presence and concentration of all amino acids at the intracellular site of synthesis determines the efficiency of anabolism. This statement was first made long before RNA was recognized as the means by which amino acid units are joined together. And so in 1951 the geometric rather than arithmetic mean of the egg ratios of the essential amino acids was proposed (1) for calculating an index of nutritive value (Table IV).

TABLE III

Egg Ratios of Amino Acids in Cereal Proteins (N × 6.25) (1)

Amino acid	Egg ratio, %						
	Wheat					White rice	Rolled oats
	Whole	Flour	Germ	Bran	Gluten		
Lysine	39	27	79	56	29	46	51
Tryptophan	80	53	67	87	67	87	87
Isoleucine	52	55	59	58	48	68	64
Valine	60	57	60	57	58	86	75
Arginine	65	59	91	114	59	109	103
Methionine and cystine	67	61	42	44	45	67	59
Threonine	77	63	147	58	63	88	84
Leucine	76	76	73	71	82	89	87
Phenylalanine	81	87	48	48	87	79	87
Histidine	88	92	104	71	92	71	96
Distribution of protein %	100	77	4	19	....	....	....
EAA index	67	60	69	63	60	77	77

TABLE IV

Calculation of EAA Index of Three Proteins (1)

Amino acid	White flour		Whole corn		Gelatin	
	Egg ratio	Log egg ratio	Egg ratio	Log egg ratio	Egg ratio	Log egg ratio
Lysine	27.1	1.4330	32.9	1.5172	71.5	1.8543
Tryptophan	53.3	1.7267	40.0	1.6021	0.0[a]	0.0000
Isoleucine	54.5	1.7364	83.1	1.9196	22.1	1.3444
Valine	56.9	1.7551	73.6	1.8669	38.9	1.5899
Arginine	59.1	1.7716	72.7	1.8615	124.2[b]	2.0000
Methionine and cystine	60.9	1.7788	71.9	1.8567	14.1	1.1492
Threonine	62.8	1.7980	86.1	1.9350	44.2	1.6454
Leucine	76.1	1.8814	163.1[a]	2.0000	38.0	1.5798
Phenylalanine	87.3	1.9410	79.4	1.8998	36.5	1.5623
Histidine	91.7	1.9624	104.8[a]	2.0000	37.5	1.5740
Sum of logs		17.7844		8.4588		14.2993
Sum of logs/10		1.7784		1.8459		1.4299
EAA index		60.3		70.1		26.9

[a] Minimum effective egg ratio = 1.
[b] Maximum effective egg ratio = 100.

These EAA indexes correlated quite well (r = 0.85) with biological values as determined by nitrogen balance studies: EAA = (0.877 × BV) + 7.38.

Mitchell (2) subsequently reported a modification of the procedure using his combination of amino acids (Table II). He claimed closer correlation with biological values and calculated a somewhat modified regression equation based on biological values in dogs and pigs as well as rats where BV = (1.074 × Modified EAA) − 13.74 and the correlation coefficient was 0.95.

Whereas the EAA index is a useful means of predicting nutritional value from an amino acid spectrum, it has certain limitations. The amino acid analysis of proteins is based on complete acid hydrolysis and in computing the index it is assumed that the protein is completely digestible and absorbed. This is more or less true within certain classes of food. Biologically, both the rate and the degree of release of amino acids during intestinal proteolysis are important. Several investigators have proposed modifications of the EAA index based on the amino acid content of enzyme digests of the protein. Sheffner et al. (5) and Mauron (6) have used such modified methods for calculating indexes, but their methods require considerably more analytical effort and time. Within certain classes of proteinaceous foods, such as fish protein concentrates or processed oil seed proteins, digestibility does not vary greatly, hence good correlation is observed between the indexes derived from EAA analysis of complete hydrolyzates and biological values.

The question of amino acid supplementation of proteins has been raised. It is well known that delayed supplementation of a protein with a deficient amino acid, e.g., wheat flour with lysine, is not as effective as simultaneous supplementation. As a result of differential rates of enzymatic release and intestinal absorption of chemically similar aggregations of amino acids, free or combined, the available pool at the site of synthesis may vary. This principle has been advanced to account for differences in utilization of analytically similar proteins or in the efficacy of amino acid supplementation (7).

A number of animal test procedures used for evaluating proteins are: (a) Protein efficiency ratio (PER) = g gain/g protein eaten. (b) Net protein ratio (NPR) = (g gain − g increment of controls)/g protein eaten. (c) Protein retention efficiency (PRE) = 100 NPR/6.24. (d) Protein nitrogen efficiency (PNE) = g gain/g N intake. (e) Coefficient of digestibility (CD or % absorbed) = 100 (N intake − fecal N)/N intake. (f) Biological value (BV or % retained) = 100 (N absorbed − urinary N)/N intake. (g) Net protein utilization (NPU) = BV × CD. (h) Efficiency of food utilization (EFU) = g gain/100g food eaten. (i) Feed efficiency (FE) = g feed eaten/g net gain. The various methods are based on either growth response or nitrogen retention resulting from feeding a standard, suboptimal level of protein. The more precise procedures estimate both the digestibility, i.e., the proportion of the nitrogen intake absorbed from the gastrointestinal tract, and the biological value of the protein, i.e., the proportion of the absorbed nitrogen retained by the body.

The most commonly used assay is the determination in rats of the Protein Efficiency Ratio (PER), i.e., the gain in body weight per unit weight of protein consumed. The test conditions are standardized for age and sex of the animals (weanling males), the level of nitrogen in the diet [10% of crude protein (N × 6.25)], the caloric density of the diet (if the test material contains a significant proportion of fat), and the duration of the test (28 days). Despite its wide usage, the PER method has several limitations which are characteristic of simple growth tests, the principal one being that it measures net growth and reveals nothing concerning the composition of the gain in body weight. Weight gain might represent retention of adipose or skeletal tissue as well as protein retention.

Observed PER values for a given test sample may vary among different laboratories with differences in the age and strain of rats, or in their pretest nutritional history. Hence the responses of test groups are compared with that of a parallel control group receiving a reference protein, casein, and the observed PER values are adjusted to an assumed value of 2.5 for the reference standard. The nutrient reserves of the animals at the start of the test period will influence the response to a poor protein more than to a high quality protein, e.g., the standard. The correction procedure is further criticized because the casein (usually ANRC casein) like most proteins tends to deteriorate in biological value with time and supplies must be replenished from time to time.

Single point bioassays provide no information concerning the slope of the response curves. Hence one of the recommendations for improving these procedures involves the use of multiple levels of both standard and unknown with tests for slope and parallelism. The slope ratio method proposed by Hegsted and Chang (8) also gives a statistically more valid assay than the single level PER determination.

Another problem is the assay of low protein products, i.e., those which cannot be included in the diet to the extent of 10%. For example, certain cereals contain only 6% or 8% of protein so that, without concentration of the protein component, it becomes impossible to include a 10% level in the diet. Here, one feeds the reference casein at a correspondingly low level.

Net Protein Utilization (NPU) is a measure of the amount of nitrogen retained by the animal under similar standardized conditions, the test period being shorter, i.e., 5 to 10 days. The nitrogen retention is measured in comparison with that lost by a control group of rats receiving no protein in the diet. In effect, NPU measures the maintenance plus the growth requirements and is equivalent to the product of the biological value and the coefficient of digestibility. These relationships and procedures for their measurement are reviewed and explained in References 3 and 9. A more sophisticated and more informative procedure for evaluating proteins nutritionally is that of Thomas (10) as adopted by Mitchell (11). This is a nitrogen balance method and requires especially careful techniques for housing and maintenance of the animals. It is a bookkeeping experiment in which output, i.e., fecal and urinary nitrogen, is compared with intake, i.e., the dietary nitrogen consumed. Animals have to be maintained in special cages to permit separation and collection of the excreta. If one subtracts from the nitrogen intake only fecal nitrogen, the difference divided by the intake is a rough measure of net utilization. To measure both absorption and retention, one has to analyze the urine as well as the feces and make corrections for metabolic and endogenous nitrogen (i.e., the fecal and urinary

TABLE V

Subacute Oral Toxicity Tests[a]

	Subacute	
	Rats	Dogs
Number of animals (minimum)		
Total	120	16
Per group	10 Males 10 Females	2 Males 2 Females
Number of groups		
Test (dosage levels)	5	3
Control	1	1
Duration of test period	3 months	3 months

[a] Adapted from FDA Program.

nitrogen, respectively, excreted on a nitrogen-free diet). It is assumed that the metabolic and endogenous excretion determined at a period when the rats are getting the no-protein (or very low protein) diet are the same as during the period when they are getting the test proteins.

A defect in the method is that it is based on testing the protein at a single level. When the level of protein in the diet is increased, the biological value or PER will rise to a maximum and then fall. An excess of even a high quality protein like casein will depress the efficiency with which it utilized, since some of the protein is utilized for energy purposes rather than for growth. Osborne et al. (12) who originally proposed this procedure recommended the maximum PER as the measure of protein quality, but since comparisons are more valid on the submaximal, rising portion of the dose-response curve, the 10% dietary level has become generally adopted.

Like the previously described methods, the biological value estimations are based on ad lib. feeding. The test in rats would be refined by underfeeding at a fixed daily food intake, for example, 10 g. In feeding studies of novel proteins, a depression in growth rate may be due not to inferior nutritional value, per se, but to the presence of a toxic substance such as a solvent or pesticide residue or some adventitious contaminant. Obviously therefore it is necessary to differentiate between nutritional imbalance or inadequacy and toxicity, especially in long term tests. Routinely, nutritional assays are rarely carried out over a longer period than a month but safety evaluations may extend over a period ranging from three months to as much as two years.

The initial phase of a toxicological evaluation consists of a series of chemical examinations designed to establish the composition and uniformity of the product so as to identify it with the product that is ultimately to be manufactured. It is poor business to undertake a two-year study on a laboratory sample if it is not representative of the end product.

Feeding studies are then done on a short term (three-month) basis in accordance with the general outline shown in Table V using relatively small groups of rats, equally divided between the sexes, and a range of dose levels. Observations are made of growth, food utilization and a large series of clinical parameters for estimating a maximum tolerated dietary level of the test material and the maximum no-effect level. Similar studies are done in small groups of dogs. In the long term (one- to two-year) tests, a similar pattern is followed, modified by the size of the groups and the frequency of clinical tests.

Testing at relatively safe dosage levels, i.e., in the range where little or no effect is observed is like looking for something that is not there. So one has to conduct a large number of more or less arbitrary tests in the search for some evidence of an adverse

TABLE VI

Typical Criteria Used in Toxicological Evaluations

Observations	Frequency studies	
	Short term	Long term
Physical appearance	Daily	Daily
Behavior	Daily	Daily
Body weight	Weekly	Weekly
Food consumption	Weekly	Monthly after 12th week
Hematology		
Hemoglobin	0,4,8,12 weeks	1,3,6,9,12,18,24 months
Hematocrit		
Leukocytes		
Total and differential		
Platelets		
Reticulocytes		
Blood chemistry		
Glucose	As above	As above
Urea N		
Protein, total		
Albumin/globulin ratio		
Triglycerides		
Cholesterol		
SGOT, SGPT		
Alkaline phosphatase		
Urine		
Volume, pH, sp. gr.	As above	As above
Glucose		
Protein		
Ketone bodies		
Bile		
Occult blood		
Sediment		
Autopsy (dead or killed animals)	Terminal	Terminal
Gross pathological examination		
Organ weights Liver, kidney, heart, brain, spleen, gonads, pituitary, adrenals, thyroid		
Histopathology		
20+ organs and tissues		
Electron microscopy of liver, kidneys and heart		

effect. As shown in Table VI, the animals are examined daily for appearance, behavior, the condition of excreta and possible neurological effects. Observations are made weekly for weight changes and food consumption.

Examinations of blood and urine in long term tests are carried out at 1, 3, 6, 9, 12, 18 and 24 months. Special tests, functional or metabolic, may be performed depending upon the toxic nature of the test material or its trace components. The liver and kidney are frequent target organs and hence are the subject of tests for functional efficiency. Diets poor in protein may cause deposition of fat in the liver or impair the capacity of the liver to produce enzymes. Animals that die or are killed at termination are carefully autopsied, organs are weighed and intensive search is made for gross evidence of malignancies or other pathological alterations. Histopathological examinations are conducted of a large variety of organs and tissues not only by light microscopy but in some instances also by electron microscopy.

The extent of these observations is, of course, influenced by the novelty of the test materials or of the process used in their production. In some tests even more intensive study is indicated. For example, reproduction experiments may be conducted through one or more generations. A schematic outline proposed by Oser & Oser (13) illustrates how the initial generation is allowed to mate and produce litters, and progeny from each successive generation are allowed to grow to maturity and produce additional litters. This may continue for two years during which period three or possibly four generations are produced.

With some products, it is necessary to consider the possibility of teratological effects, that is the congenital malformations that were made so notorious by the thalidomide disaster. This requires reproduction studies of a special type in which the time of the

administration of the potentially toxic agent to rats or rabbits is regulated in relation to the pregnancy. Some groups are dosed before mating and some during stages of gestation. The animals are generally delivered by Caesarian section and examined for implantation sites and resorptions and the pups are carefully examined for soft tissue and skeletal deformities.

Potential carcinogenicity may also be a factor in certain types of products. For example, in single cell proteins derived from a petroleum source, one would think of polycyclic aromatic hydrocarbons. This would require not only very careful chemical analysis of the protein product, but observation of animals over long periods to determine whether any latent effect is induced by subdetectable levels of the hydrocarbon.

Lately there has been a good deal of speculation upon possible mutagenic effects, i.e., the induction of chromosomal aberrations which might cause genetic damage. Most of this work is presently based on in vitro studies and what it may mean in terms of the intact animal still has to be established.

REFERENCES

1. Oser, B. L., J. Amer. Dietet. Assoc. *27*, 396–402 (1951).
2. Mitchell, H. H., in "Methods for Evaluation of Nutritional Adequacy and Status," Committee on Foods, National Academy of Sciences—National Research Council, Washington D.C., 1954, p. 13–28.
3. Food and Nutrition Board, "Evaluation of Protein Quality," National Academy Sciences—National Research Council, Washington D.C., 1963, Publication 1100.
4. Mitchell, H. H., and R. J. Block, J. Biol. Chem. *163*, 599–620 (1946).
5. Sheffner, A. L., G. A. Eckfeldt and H. Spector, J. Nutr. *60*, 105–120 (1956).
6. Mauron, J., in "Evaluation of Novel Protein Products," Edited by A. E. Bender, R. Kihlberg, B. Lofqvist and L. Munck, Pergamon Press, New York, 1970.
7. Melnick, D., B. L. Oser and S. Weiss, Science *103*, 326–329 (1946).
8. Hegsted, D. M., and Y. Chang, J. Nutr. *85*, 159–168 (1965).
9. Munro, H. N., and J. B. Allison, "Mammalian Protein Metabolism," Vol. 2, Academic Press, Inc., New York, 1964.
10. Thomas, K., Arch. Anat. Physiol. 219–302; Chem. Abstr. *4*, 1331 (1909).
11. Mitchell, H. H., J. Biol. Chem. *58*, 873–903 (1924).
12. Osborne, T. B., L. B. Mendel and E. L. Ferry, Ibid. *37*, 223–229 (1919).
13. Oser, B. L., and M. Oser, J. Nutr. *60*, 491 (1956).

[Received June 30, 1970]