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PROTEIN AS HUMAN FOOD AND DETERMINATION OF ITS NUTRITIVE VALUE (PROTEIN QUALITY)

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Using present rate of increase in population as a parameter, population in the developing countries is expected to double in 20 to 30 years and in the developed countries in 80 to 90 years. Africa, Asia and Latin America alone will possibly account for 80 per cent of the world population in the year 2000. The industry of the world's supply of food protein is evidenced by widespread protein-calorie malnutrition in some developed countries resulting in high death rate (20 to 30 per cent) among the children before they attend their fifth birthday, and causing retardation in mental and physical development. Portein-calorie malnutrition is also common among pregnant mothers and elderly people of the developing countries. While the protein requirement for India and Brazil will increase by 80 to 110 per cent, for Africa by 110 to 120 per cent, for Pakistan by 120 to 150 per cent over the next 25 years, it will increase by only 40 to 50 per cent in the developed countries during the same period. However, in all these calculations no account of protein quality has been taken into consideration. The protein requirement for an adult of 70 kg body weight varies from 30 grams per day for high quality protein foods (such as milk and eggs), to 80 to 100 grams per day for poor quality plant proteins.

Protein is essential for growth, reproduction and maintenance. Its primary function is to furnish a mixture of amino acids for the synthesis of functional and structural proteins. The efficient utilization of dietary protein depends among many factors on protein quality, species, physiological conditions, and is also influenced by age and sex. Amino acids are the building blocks from which the tissue proteins are synthesised. Protein supplies the amino acids. It is also the only source of those amino acids which the body cannot synthesise (essential amino acids). For maximum utilization of amino acids they must be available at the same time at the site of protein synthesis in the correct proportions because it has been demonstrated that supplementation becomes ineffective if lysine is added to a low lysine diet after a delay of three hours when five of the ten essential amino acids have been given to rats one hour after the first five, the rats failed to grow [1, 2]. Excess dietary leucine depresses growth because it reduces food intake and impairs iso-leucine and valine utilisation [3]. Methionine requirement for greatest efficiency of feed utilization is higher than the requirement for maximum growth (4 to 6). The efficiency of protein utilization thus depends, among many factors, on overall balance of dietary amino acids. In formulating the protein component of diets, it should be remembered that incorrect or improper supplementation is not only a waste of expensive supplement but also introduces the hazard of amino acid imbalance [7].

The worldwide shortage of soyabean meal, fish meal and other lysine-rich and/or protein-rich products and their increased costs have limited their use as chief sources of protein in animal feeds in both the developing and the developed countries. Cereals which supply more than 50 per cent of the dietary protein in the developing countries, are mostly used as the source

of protein and energy in animal feeds in the develcped countries. Although widely used, they are analyzed mainly for protein content and seldom for amino acid composition and rarely assayed for digestibility and amino acid availability. Another source of plant proteins which has recently received much attention is the legumes. They are rich sources of protein, containing 20 to 35 per cent crude protein depending on the species. Also they are capable of fixing from the atmosphere much, if not all, of the nitrogen required for their growth. They are widely used for human consumption in the developing countries as well as by a large segment of the population in the developed countries.

Laboratory Methods for Protein Evaluation

The selection of a protein for a diet depends on two important characteristics of the protein: one is its potential hutritive value and the other is its true nutritive value. The former is dependent on its amino acid composition and the latter on the digestibility and amino acid availability of the protein. The amino acid composition of the various protein components of the diets and the rates of their release from proteins when digested *in vivo* by pancreatic proteases and peptidases in the lower intestinal tract, are important factors among many others that govern the growth of animals. It has been demonstrated that the most limiting amino acid in protein regulates protein biosynthesis and thereby controls the growth of the animal. It thus seems essential to determine protein contents, amino acid composition, digestibility and amino acid availability of the dietary proteins for the proper assessment of their potential and true nutritive values.

Procedures for the determination of amino acid composition of proteins

(a) Acid hydrolysis: The first requirement in the assessment of the quality of a dietary protein is the determination of its amino acid composition. The procedure which has been most widely used consists of heating the protein with 6N HCl in a sealed tube at 110°C for 24 hours. Under these conditions of digestion some destruction of serine and threonine occurs whereas the branched-chain amino acids are not completely liberated [8, 9]. To overcome this limitation, acid hydrolysis should be carried out for 12 hours, 24 hours and 72 hours and the results should be extrapolated to 0 hour [10]. This provides more realistic values for the amino acid composition of the protein. To prepare a protein hydrolysate truly representative of the amino acid composition of diets low in protein and high in carbohydrate, still remains a problem. The hydrolysis of the protein in such diets should be carried out in a large volume of HCl, viz. 10 to 12 mg protein in 250 ml 6H HCl. The use of a large excess of acids also reduces humin formation [10, 11]. Extraction with dilute HCl, formic acid or phenol, or digestion with amylase prior to acid hydrolysis, has often been beneficial for the removal of carbohydrates [9]. Extraction of fat with petroleum ether has also been found beneficial [9]. These steps should be included in the procedure where the sample is rich in carbohydrate and fat but low in protein.

(b) Alkaline hydrolysis and performic acid oxidation: Acid hydrolysis destroys tryptophan and converts methionine to methionine sulfoxide [8]. The procedure of hydrolyzing proteins with 3.5 per cent perchloric acid does not destroy tryptophan but has not been found quite suitable for the hydrolysis of proteins in diets rich in carbohydrate [13]. For the determination of tryptophan in dietary proteins they should be hydrolozed with

5N $BaSO_4$ at 120°C for 16 hours (10 ml for 100 mg protein) in a sealed tube [14] followed by the determination of the amino acid in the hydrolysate. A colorimetric method developed by Spies and Chambers [15] can also be conveniently used for tryptophan determination. This requires predigestion of the dietary protein with papain [16] or pronase [17]. Quite reproducible results can be obtained, once one becomes familiar with the method. It is also easy to perform. To determine methionine level in a protein, methionine sulfoxide must be estimated along with methionine remaining uncoverted. It has been often found advantageous to hydrolyse the protein in the presence of a reducing agent (mercaptoethanol) to convert methionine sulfoxide to methionine [18]. This eliminates the measurement of methionine sulfoxide. Sulphur amino acids can also be determined after performic acid oxidation of the protein as cysteic acid and methionine sulfone [19].

(c) Determination of amino acids in the protein hydrolysate: Recently the Food and Agricultural Organisation of the United Nations (FAO) published an extensive compilation of amino acid values in foods measured by chromatographic and microbiological methods [20]. The chromatographic method is simpler but makes no distinction between natural and unnatural isomeric forms of amino acids nor is it affected by the factors that influence the complex biological systems. However, because of the simplicity of the ion exchange column chromatographic method and the introduction of automatic amino acid analysis, all other methods have now virtually been replaced by ion exchange chromatography in countries who can afford it. Other methods are also used but only for specific reasons.

Evaluation of protein quality

The biological value (B.V.) of a protein can be defined as that percentage of absorbed protein that is retained by the body. The various biological methods that have been developed over the past 20 years to determine protein quality include: Net Protein Utilization (N.P.U.), Protein Efficiency Ratio (PER) and Plasma Amino Acid Levels (PAAL). The biological methods suffer from various disadvantages. The results obtained by such methods depend on the limiting amino acid and provide no information about other amino acids which may be present in relative excess or level as the limiting amino acid and therefore do not allow any prediction of the effects of mixing different proteins of different amino acid compositions. The biological values depend on the levels and amounts of proteins in the diet, levels of energy provided by the diet and also on the presence of other dietary factors. The biological methods may also provide misleading results if the experimental conditions are such so as to influence the amount of food eaten by the animals. Besides these, animal feeding trials are expensive and time consuming and therefore are not often suitable in the food manufacturing industry to be used for quality control, commercial development of new protein foods and rapid screening of new protein sources. Some of the laboratory methods recently developed have much more to offer and, hopefully, will replace biological methods more and more in the near future. In fact they are becoming increasingly indispensable to industry. Any practical procedure for determining the protein quality of foods and feeds must be simple, rapid, inexpensive, reasonably accurate and applicable to a wide range of dietary proteins.

(a) Chemical score method: The amino acid composition of a food protein is of utmost importance in determining its nutritional quality although amino acid composition represents only the potential nutritive

value. The Chemical Score (C.S.) method, based on the determination of the amino acid composition of the protein, was introduced some years ago by Block and Mitchell 21 as the basis of chemically evaluating protein quality. The method also requires the determination of the amino acid composition of a standard protein for comparison. The C.S. is the relative quantity of the most limiting amino acid in the protein compared to the quantity of the same amino acid present in an ideal protein, viz, egg protein. The C.S. of the protein is taken as the lowest ratio and the value is expressed relative to egg protein with a score of 1.0. The lower the ratio, the poorer is the protein quality and the higher the ratio, the better is the protein quality. Table 1 shows the chemical scores of a variety of foods and their biological values as determined by N.P.U. method. It also includes the first limiting amino acid in each of these proteins.

Table 1. Comparison of Chemical Score with Biological Value

Protein	C.S.	B.V.	Limiting amino acid
Dried milk	0.72	0.80	Sulphur aa
Dried fish	0.69	0.67	Sulphur aa
Coconut protein I	0.56	0.66	Sulphur aa
Cottonseed flour I.	0.68	0.65	Sulphur aa
Maize meal	0.50	0.35	Lysine
Yellow pea flour	0.43	0.43	Sulphur aa
Mixture, maize and pea	0.72	0.70	Sulphur aa
Bread	0.38	0.50	Lysine
+ lysine	0.66	0.61	Threonine
+ lysine + threonine	0.87	0.80	Sulphur aa
+ lysine + threonine + methionine	0.82	0.85	Valine
+ lysine + threonine + methionine + valine	-	0.93	-

A 1:1 relationship is seen for materials with B.V. greater than 40. Considerable variation between B.V. and C.S. of materials with low N.P.U. values has been reported [22]. Net protein utilization is the basis for the determination of B.V. of proteins and is based on nitrogen retention in the whole body and can be measured by direct carcass analysis or N balance techniques. The B.V./C.S. curves become almost horizontal at three different points depending upon the nature of the limiting amino acid in the protein. At lower B.V. values (<40), C.S. depends on the nature of the limiting amino acid e.g. if lysine is absent, C.S. is zero although its B.V. can be 40. If on the other hand, tryptophan, threonine, histidine, phenylalanine, leucine or isoleucine is absent, C.S. is zero but B.V. can still be 20 whereas in the absence of valine or sulphur amino acids, C.S. and B.V. (growth assay with rats) are both zero. This, however,

does not minimise the value of the C.S. method because materials with N.P.U. values greater than 40. According to figures published by FAO only 9 out of 133 values listed for different foods fall below 40 [23]. The reason why the relationship deviates by B.V. below 40 is possibly due to differences between the need of different amino acids and different amounts of the amino acids for different purposes, e.g. growth and maintenance. An N.P.U. value of 40 approximately equals a Protein Efficiency Ratio (PER) of zero, i.e. maintenance but not growth [24].

The procedure for using C.S. to predict B.V. is based on chemical analysis of the test protein for amino acid composition. The relationship between C.S. and B.V. as shown in Table 1 was obtained by different workers under standardized conditions, using mixtures of amino acids and proteins of known compositions which were assayed biologically. This allows the prediction of the B.V. of the protein from its C.S. determination.

(b) *Microbiological assay:* Microbiological assay measures the growth responses of an organism to graded supplements of the test protein and compares this with responses obtained from the graded doses of the limiting amino acid in a solution of amino acids simulating the amino acid contents of the test protein minus the limiting amino acid. The organisms, widely used for the purpose are *Streptococcus zymogenes* and *Tetrahymena pyriformis* whose amino acid requirement patterns resemble most closely the amino acid requirement patterns of higher animals [25]. However, some important differences have been reported viz. *Streptococcus* does not require lysine for growth, whereas serine, a non-essential amino acid for higher animals is essential for *Tetrahymena* [25]. The enzymatic predigestion of the protein in the diet or feed with papain appears to be essential, in order to obtain results more compatible with the values obtained from Carpenter's dinitroflurobenzene method and rat growth tests (25 to 28) as shown in Table 2. The predigestion with papain largely eliminates the most laborious and time consuming microscopic counting of the cells in the test cultures. This also minimises interferences with optical density measurement which is considered as directly proportional to the number of cells present. To make the procedure easier to perform, the test cultures could be grown in Pyrex tubes. The products of enzyme digestion should be diluted to contain 0.3 mg N/ml for optical density measurements as recommended by Shorrock and Ford [28]. Five different proteins, including soyabean and groundnut were assayed by the Tetrahymena method for their available lysine. A fairly close agreement between the results was obtained from direct microscopical counts and measurements of optical density, except in the case of groundnut meal where discrepancy was noted [28].

Tetrahymena assay requires enzymatic predigestion for 96 hours while for Streptococcus assay, 48 hours predigestion is sufficient. The latter is more suitable for samples rich in carbohydrate because growth can also be assayed in this method from the results of measurements of the acid produced during growth of the test cultures [28]. However, Streptococcus assay can neither be used to measure lysine content in the protein nor lysine availability. It is more suitable for the determination of methionine and its availability. Tetrahymena assay offers the advantage of measuring both lysine and methionine in the same test extract [28]. Streptococcus assay is simpler and more reliable than the other, especially if the diet is rich in carbohydrate and low in protein. Microbiological assay is very useful where automated amino acid analyzer is not available; with proper training one can obtain fairly reproducible results.

Table 2. Available Lysine Values (g/16 N) for Different Food Proteins Determined Microbiologically with Tetrahymena before and after Digestion with Papain, Chemically by FDNB Reaction and Rat Growth Assay.

	Tetrahymena			
	No digestion	Pre-digestion	FDNB	Rat growth assay.
Fish meal (FM 101)	4.8	7.0	6.1	6.9
Meat meal (MM 101)	1.1	3.1	4.1	4.3
Whale meat meal (WM 1)	1.2	3.6	4.1	4.2
Hydrocarbon yeast (HY 101)	1.9	5.9	5.7	7.0
Groundnut meal (GN)	2.1	3.0	3.6	3.2
Groundnut meal, (heated HGN)	0.6	1.7	1.3	1.0
Soyabean meal (SB)	4.2	4.4	5.4	5.0
Soyabean meal (heated HSB)	1.0	2.4	2.4	1.3
Cod muscle (CM)	6.4	8.6	8.5	10.9
Cod muscle (heated HCM)	1.0	5.0	5.6	4.3
Casein	-	8.1	8.4	8.6

Source: Scott, J.A. and Smith, H. (1966). *Br. J. Nutr.* 20, 663-673.

(c) Dinitroflurobenzene procedure for the determination of available lysine: Under usual circumstances, lysine, methionine or both, are the limiting amino acids in conventional animal diets. It is therefore important for the feed industry and farmers to know how much, in addition to standard inclusion, should be added to a low lysine diet to optimise performance or what dilution can be made if it is of above average quality. It has been suggested that lysine that does not react with dinitroflurobenzene (DNFB) is not nutritionally available even though it is included in the conventional measure of total lysine in acid hydrolysates [26]. A measure of available lysine will reflect the nutritional value of the dietary protein. This initiated a search for a suitable laboratory method for the determination of available lysine in proteins. The procedure developed by Carpenter [26] and modified by Baliga *et al* [28] has been successfully employed in recent years in determining the quality of proteins of animal origin [26, 29, 31]. It has also been found to be quite useful in detecting losses of nutritive values due to processing (heat damage) and storage [31-33].

The procedure developed by Carpenter [26] is based on the Sanger's reaction, in which lysine residues with free ε-NH_2 groups in the protein are allowed to react with dinitroflurobenzene (DNFB) for 2 hours in $NaHCO_3$ medium and then subjected to 16 hours reflux in 5.8 N HCl on an oil bath. The coloured DNP -amino acids found in the acid hydrolysate are: ε-DNP

lysine, α-DNP arginine, α-DNP ornithine, ε-DNP hydroxylysine. The acid hydrolysate is subjected to ether extraction which removes all coloured DNP derivatives except ε-DNP lysine and α-DNP arginine. This is followed by treatment with methoxycarbonyl chloride (MCC), reacidifying and extracting with ether. This removes ε-DNP lysine. The ε-DNP lysine is estimated by subtracting the colour of the acqueous layer after treatment with MCC and reextraction with ether from the colour remaining after first ether extraction of the acid hydrolysate. During acid hydrolysis some destruction of DNPL occurs. Carpenter introduced a recovery factor based on the percentage of recovery of a standard ε-DNPL added to the sample after DNFB reaction but prior to acid hydrolysis in a second experiment. Using the recovery factor it is possible to calculate available lysine in the protein.

Modification of Carpenter's method

In the original Carpenter's method the recovery of ε-DNP lysine (DNPL) was found to be 90 per cent or more for proteins of animal origin and 60 to 85 per cent for plant proteins depending upon the carbohydrate content. It has been found that the higher the carbohydrate content in the sample the lower is the recovery. Since the recovery of the added DNPL provides the correction factor by which the results are to be multiplied in order to get the correct loss incurred during acid hydrolysis, the final calculation greatly depends on this recovery factor. Besides the destruction of DNPL during acid hydrolysis, it is also adsorbed on the residues. To minimise the latter loss, Booth [34] recommended that the acid hydrolysate was to be filtered hot and the residue should be washed thoroughly. He also pointed out that the hydrolysis of dinitrophenylated protein is never completed after 16 hours reflux in 5.8 N HCl but cannot be continued for a longer period because hydrolysis for more than 16 hours causes greater destruction of DNPL. To avoid this, a second hydrolysis has been recommended. This also eliminates the use of a large volume of acid to hydrolyse a sample rich in carbohydrate and low in protein. Booth [34] also found that the loss of DNPL in protein due to destruction during acid hydrolysis was less than that found with DNPL when added as such before acid hydrolysis. In fact, the loss of dinitrophenylated protein when used instead of DNPL as a recovery agent, was reduced by almost 50 per cent. Using this modification, Booth found from the results of 21 determinations on cereals, legumes, fish meals and meat meals that better correlation could be obtained with materials that contain no or very little carbohydrate such as animal proteins, if the results were to be multiplied by 1.05 instead of 1.09 as suggested originally by Carpenter. In the case of cereals, the results should be multiplied by 1.2 and the results from legumes, by 1.14. The introduction of these correction factors, makes the Carpenter's method more valuable and reliable and can be recommended for wide application in industry. The Silcock method, developed by Roach, Sanderson and Williams [35]measures lysine before and after treatment of a sample with DNFB; the latter lysine being that which is inaccessible to DNFB. This method is more specific, it includes N-terminal lysine as well as free lysine but excludes all other amino acids. Carbohydrate does not affect the results nor does humin form during acid hydrolysis which has been always a source of error in Carpenter's method. However, it requires complex apparatus and specialized skill to perform. This limits its routine use for protein evaluation work. The Silcock method is more suitable for research. Carpenter's method on the other hand, is simpler and cheaper and, when suitable correction factors are introduced, gives results which correlate well with gross protein values.

(d) Methods based on measurements of amino acids released from dietary proteins when subjected to enzymatic hydrolysis in vitro: It has been generally agreed that there is a real need for reasonably good methods for evaluation of protein quality of diets, foods, feeds and plant proteins which vary considerably in quality. The supply of good quality protein (amino acids) takes a considerable proportion of the cost of a balanced diet (20 to 30 per cent for fast-growing species). The recognition in recent years of amino acid availability as an importer criterion for protein evaluation, initiated the development of a simple laboratory method, based on measurement of amino acid composition and the rates of release of amino acids from the dietary food and feed proteins during digestion by pancreatin *in vitro* under specified conditions. Melinik and Oser [36] first suggested that the determination of amino acid composition of a protein and the measurement of its susceptibility to enzymatic digestion might be useful in the evaluation of protein quality. The usefulness of enzymatic hydrolysis *in vitro* in assessing the true nutritive value of a protein, has also been pointed out by other workers [9, 36-40].

Shaffner and co-workers [41] developed a method based on the measurements of essential amino acids released from dietary proteins after hydrolysis by pepsin *in vitro* and the amino acids released from the undigested protein after acid hydrolysis. They used microbiological assays to determine the concentrations of individual amino acids and expressed the results as pepsin-digest-residue (PDR) amino acid index. Despite some merits, the method was never used in assessing protein quality of feedstuffs of foods, because of limited hydrolysis of proteins by pepsin and estimation of individual amino acids by microbiological assays required considerable work and time.

It is practically impossible to replicate precisely *in vitro* the hydrolytic processes that are occurring *in vivo* in the intestinal tract but the information regarding amino acid availability of the protein may be obtained from such studies and might be useful protein evaluation. If the primary objective is to know the rates of release of amino acids from a dietary protein during digestion *in vitro*, it is more desirable to use an enzyme preparation which resembles closely the enzymes present in the pancreatic juice. It has been known for some time that animals deprived of pancreatic secretion are unable to utilize proteins efficiently. In the intestinal tract, food proteins are exposed to the action of a series of endo and exo-proteases and peptidases. The results of the experiments with pancreatin (a polyvalent enzyme mixture) *in vitro*, suggest that pancreatin when used in a relatively high concentration, can digest proteins fairly extensively and can provide useful information for protein evaluation [9]. Akeson and Stahmann [40] intorduced some modifications of the PDR amino acid index method of Shaffner *et al* [41]. This involves digestion of the protein by pepsin at pH 2.0 in 0.1 N HCl acid medium for 16 hours at 30°C. After neutralization to pH 7.4, it is digested with pancreatin for 48 hours. Aliquots, are removed at different intervals and analyzed for the concentrations of individual amino acids released. The duration of hydrolysis and the reagents to be used for terminating the reaction are also important, because if small peptides are not properly precipated they interfere with the determination of amino acids. Because different amino acids are released at different rates during protein digestion, hydrolysis should be carried out for different lengths of time. The amino acids released can be determined in an automatic amino acid analyser. Akeson and Stahmann [40] measured amino acids released during pepsin-pancreatin digestion of 28 different food proteins of animal and plant origins and found an excellent agreement between their results on protein quality and published biological

values for growing rats. Saunders and Kohler [42] measured protein digestibility in wheat mill feeds using the pepsin-pancreatin digestion method of Akeson and Stahmann and observed a good agreement with the results of protein digestibility measurements with the results obtained from rat feeding trials. Recently Saunders and co-workers [43] measured digestibility of various alfalfa protein concentrates using Akeson and Stahmann's method and found a high degree of correlation with these results and those obtained from rat feeding trials.

From the comparisons of the results obtained by various laboratory methods for protein quality of different foods and feeds based on the determination of chemical score, available lysine (Carpenter's method), available lysine and methionine (microbiological assays), digestibility and amino acid availability (pepsin-pancreatin-digest amino acid index method) to the gross biological values obtained by using NPU and PER methods, it becomes apparent that some of the laboratory methods could be used in industry to predict the biological values of proteins (in foods and feeds) and to detect the loss of nutritive values due to processing and storage as well as for screening new protein sources.

Conclusion

A large segment of the world's population does not receive adequate protein. Because of the general shortage of protein-rich foods such as meat, eggs, and dairy products which are also high-priced, a large segment of the world's population does not receive adequate animal protein. Protein requirements for the next decade will be greatly increased and hopefully can be met by enriching cereals, legumes, leaf proteins with the amino acids in which they are deficient, by genetic improvement of corn and other grains for the people of the developing countries, and by increasing the production of very high quality animal proteins for only those who can afford them. The concept of an enrichment of low quality protein to meet the challenge of feeding the populations of the developing countries of the world with adequate amounts of balanced proteins, requires the development of rapid, inexpensive and reasonably accurate laboratory methods for the determination of the quality of dietary proteins in view of the many advantages the laboratory methods can offer to feed manufacturing industries. All potential sources of food protein must be explored and developed to meet the world's need. In many of the Caribbean countries there might be barriers to increased production of agricultural products because of the absence of enough established chemical industries to produce fertilizers and food processing industries. There is also lack of trained personnel at all levels who are able to deal with food problems involving production, processing, preservation and evaluation of food proteins from different sources. Efforts should be directed both by the Government and the University to establish a Central Institute of Food and Agriculture to deal with the problems.

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