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Evaluation of protein quality: Methodological considerations

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It is 25 years since the Society held a meeting on this same topic (12 October 1957). The enormous efforts that have gone into protein evaluation in the intervening quarter century suggest a need to reiterate and emphasize points made at that meeting (Bender, 1958) namely, that all measures of protein quality are a function of the limiting amino acid (AA), that the tests do not yield any information about other AA and that there is no 'true' value with which to compare new methods. Protein efficiency ratio (PER) has been used for too long as the touchstone of protein quality evaluation and it is time to abandon it.

The fundamental error underlying all this work is the attempt to express protein quality by a single figure. Such an attempt might have been justified when biological measures were first developed because at that time it was not known that the value was dependent on the AA in the food—in fact, the major methods were in use before the last of the essential AA, threonine, was discovered. The term NDpE is used in which both quality (actually the amount of (unspecified) limiting AA) and quantity are incorporated. NDpE does not indicate whether there is insufficient protein in the diet, whether more food of the same type would be beneficial or whether the quality of the protein is low and supplementation is the solution.

Evaluation of protein quality

The term 'bioassay' in relation to protein quality is somewhat misleading. This term is usually applied to assays in which there is dose-related response, such as in pharmacological assays and in many assays using micro-organisms. The validity of such tests is often demonstrated by the slope of the line.

The classical protein quality evaluations (PQE), however, despite occasional statements to the contrary, are not dose-related but measure simply how much of the protein is retained by the animal, whether estimated from N retention or

weight gain with allowances for maintenance. So it is probably clearer to refer to this as the evaluation of protein quality rather than a bioassay.

The 'best' method of PQE depends on the use that will be made of the findings. The protein under test may be intended as the sole food of an infant, as a supplement to the mixed diet of an adult, or that of a pregnant or lactating woman. It may be intended to replace another source, it may be used to supplement animal diets for the production of eggs, milk, meat or wool, or the problem may be to assess processing changes. The method serves to provide only an index for the value of the protein for one of these purposes.

Methods used

The search for screening methods, short cuts and rapid methods continues and each is almost invariably compared with PER as the standard. Some of the traditional, as well as the newer methods, are listed in Table 1.

Table 1.   Some methods of measuring protein quality

NBI (nitrogen balance index): Tangent of the curve relating N balance to absorbed N
     (numerically similar to BV) (Allison & Anderson, 1945)

NGI (nitrogen growth index): Slope of the line relating weight gain to N intake
     (corresponds to NBI and NPR) (Allison, 1959)

NU (nitrogen utilization value): Weight change in 14 d plus 10% of initial and final
     weights to allow for maintenance N (McLaughlan, 1976)

RNU (relative N utilization value): NU expressed as percentage of lactalbumin
     (McLaughlan, 1976)

NPR (net protein ratio): Weight gain of test group plus weight loss of non-protein
     group divided by protein consumed (Bender & Doell, 1957)

RNPR (relative net protein ratio): NPR compared with reference protein (McLaughlan
     et al. 1980)

RPV (relative protein value): Regression line relating dose to response for test protein
     compared with reference protein omitting zero protein level (Samonds & Hegsted,
     1977)

PPV (predictive protein value) (Gross & Gross, 1980)

CPE (complete protein evaluation) (Schelling, 1975)

Formol titration (Petit-clerc et al. 1980)

Rapid biological methods

Red flour beetle (*Tribolium castaneum*) (Medrano & Bressani, 1977)
Confused flour beetle (*Tribolium confusum*) (Sharma et al. 1977)
Aspergillus flavus (Moohyuddin et al. 1978)
Leuconostoc mesenteroides (Hannah et al. 1977)
Insects (Loschiavo, 1980)
E. coli (Bell et al. 1977)
Tetrahymena, modified (Baker et al. 1978)
Mealworm (*Tenebrio molitor*) (Davis, 1975)

Abbreviated indices

Plasma AA index (Whitaker & Patrick, 1971)
Blood urea concentration (Münchow & Bergner, 1968)
Blood arginase, ornithine carbamyl transferase and glutamic—pyruvic transaminase
     (Bergner, 1977)
Blood catalase (Kirschgessner et al. 1977)
Ribosomal incorporation of AA (von der Decken et al. 1975)

Much effort has been devoted to abbreviated methods and since so many protein sources are limited by either lysine or S-AA many misleading correlations have been claimed. If a chemical method measures, for example, amino groups and thus, largely, available lysine, then it will certainly correlate with PQE for proteins limited by lysine. If a small number of the proteins in the test group are limited by a different AA they are outliers but there will still be a high correlation between methods.

Boyne *et al.* (1961) found that protein quality index correlated well with gross protein value (GPV) (which depends on lysine) for a group of whale meals, but not with GPV or net protein utilization value (NPU) for a group of meat, cotton-seed or soya-bean meals, mostly limited by S-AA. The same authors showed that for some proteins there was even a correlation between simple solubility and biological PQE. It is sometimes possible to use such simple chemical indices to rank proteins of one particular type but there is no true basis for any correlations found and the methods can certainly not be used to compare proteins from different sources.

Some correlations between chemical and biological indices have been shown on a small number of samples and later disproved when larger numbers were tested. The earlier method of chemical measurement through copper precipitation (Almquist *et al.* 1935) correlated well with a biological method on the six proteins examined, but subsequent work on other proteins discredited the method. If there is no theoretical basis for a method then correlations are not reliable indices of validity. Schelling *et al.* (1980) claimed to demonstrate the value of the complete protein evaluation (CPE) method by studying just two proteins.

Errors of principle

A number of misconceptions have crept into official publications and have thereupon become enshrined in the mysticism of protein quality. The Protein-Calorie Advisory Group (PAG) (Anon, 1974) pointed to (some of) the limitations of PER and NPU (by carcass analysis) and stated categorically that 'for a more definitive and detailed appraisal of the nutritive value of a protein source, nitrogen balance procedures must be used'. NPU, determined by carcass analysis, with digestibility (D) allows the estimation of biological value (BV) and these are fundamentally the same measures as BV and D measured by the lengthier procedures of N balance which, of course, allows the estimation of NPU.

The PAG recommendations state that diets fed *ad lib.* introduce an error inasmuch as the ratio of weight gain to food consumption may be influenced by the latter. Table 2 shows that this is true of PER but not for the other two methods shown, both theoretical and experimental.

The PAG report also states that the PER method could be improved by feeding at different levels, as indeed was done in the original procedure of Osborne *et al.* (1919) but, if low levels of dietary protein satisfy only maintenance requirements while higher levels also satisfy growth requirements, such a procedure might result only in further confusion by changing the limiting amino acid. Campbell &

Table 2. *Effect of food intake on PER in rats*

*Theoretical calculation**

Protein eaten (g)	Change in body-wt (g)	PER	NPR
0	−10	—	--
1	−5	—	$5/1 = 5$
2	0	$0/2 = 0$	$10/2 = 5$
3	5	$5/3 = 1.7$	$15/3 = 5$
4	10	$10/4 = 2.5$	$20/4 = 5$
5	15	$15/5 = 3.0$	$25/5 = 5$

Experimental results (10-d assay)

Protein eaten (g/100 g body-wt)	PER	NPR	NPU
Dried skim milk			
11.9	1.21	3.60	63
12.6	1.70	3.86	64
13.8	2.47	3.80	64
16.3	2.68	3.80	63
16.3	2.76	3.74	61
17.3	2.77	3.74	64
Bread fortified with lysine			
6.6	0.17	3.00	59
7.2†	0.63	3.14	57
7.4	0.85	3.32	56
11.0	1.81	3.34	54
14.7	2.28	3.74	57

*Assume 2 g protein required for maintenance of weight and 1 g protein retained produces 5 g weight increase.

†7-d Experiment: protein intake multiplied by 10/7. All other figures from 10-d experiments.

McLaughlan (1971) showed the NPU of an AA mixture, formulated to suit maintenance, to be 88 when fed at 2.5% of the diet (maintenance level) or 41 when fed at 9.5%. Kies & Fox (1970) found that the second limiting AA in maize for man was tryptophan when 4 g were fed and methionine when 13 g were fed at the same intake of essential AA.

Time to abandon PER

A standardized PER procedure was adopted in Canada (Chapman *et al.* 1959) and in the US (AOAC, 1960) and subsequently almost every proposed new method of assessing protein quality has been compared with PER (Landers, 1975; Evanco *et al.* 1977; Satterlee *et al.* 1979; Pellet & Young, 1980). This has placed PER values in a position of being regarded as the ultimate and 'true' measure of the quality of a protein—a position which is quite unmerited and which has been criticized many times. However, it still remains the touchstone of protein assay. Unless the newer methods correlate or agree with PER then they are disregarded.

This position is invalid for the following reasons:

1. PER depends, as indeed do all the biological methods, only on the limiting AA and takes no account of the other AA.
2. PER varies with the amount of food eaten.
3. The weight gain is assumed to be protein tissue but can include large and variable amounts of fat which can give erratic and incorrect results.

Variation of PER with food intake

It has long been known that PER increases with protein fed and therefore total diet consumed (Mitchell, 1924, 1944; Stewart *et al.* 1943; Barnes *et al.* 1945). The theoretical calculations in Table 2 have been repeatedly confirmed in practice. An example of the errors that arise was the report of Shyamala & Kennedy (1962) that cooking improved the nutritive value of wheat protein, but Milner & Carpenter (1969) showed that this result was incorrect and due entirely to apparently increased palatability of the cooked wheat protein to the rat. The inclusion of a standardized casein diet and correction of results to a PER value of 2·5 for casein does not help if the test diet is particularly palatable or unpalatable to the rats. It is not clear why different batches of rats, even from the same colony, should vary so much in their food intake but it does emphasize the futility of carrying out assays without duplicating them.

This is well illustrated in an experiment (A. E. Bender, unpublished results) in which all the animals under test behaved in a very abnormal fashion. A number of preparations of meat and mixtures of meat and soya were assayed for PER by the standard AOAC (1960) method at a level of 10% dietary protein. After 7 d the results for all groups reached the impossible value of PER 5. Since 4·5 g of muscle tissue contain 1 g protein the maximum possible PER is 4·5. After 14 d the value had reached 6·0. At this stage the animals were sacrificed and the carcasses analysed for fat content by extraction with petroleum ether. On the meat diets three animals contained 39·3, 35·8 and 41·8% fat expressed as percentage of total dry weight of carcass, whereas the values usually obtained with rats from the same colony were 20–30%. All forty animals in the batch of rats used in this experiment laid down similarly large amounts of body fat, so it would seem that even if the experiment had been carried out on larger groups of animals from the same batch the same incorrect values would have been obtained.

Before and after the adoption of PER as the official method for the assessment of protein quality it was concluded that the results from ten laboratories showed a high degree of agreement (Derse, 1960, 1962). This, however, is not supported by the experimental results (Table 3).

PER has often been preferred to BV and NPU (carcass method) because of its apparent simplicity and low cost. Relative costings have been published (Bodwell, 1977). However, Reaidi (1981) found that PER (not duplicated but carried out by the AOAC procedure) required forty animals in four cages and took 28 d and 43 man-hours; NPR required thirty-two animals in eight cages and took 4 man-hours;

Table 3. *Variation between laboratories in PER estimation of a number of foods, as observed and after correction for casein standard (from Derse, 1960, 1962)*

	Coefficient of variation	
	PER as observed	PER corrected for casein at 2·5
Casein	15·4	—
Soya-bean meal	8·9	19·9
Egg	7·7	18·2
Wheat flour	26·8	26·5
Soya-wheat flour mixture	4·9	20·1
Egg-wheat flour mixture	8·2	17·2
Casein-wheat flour mixture	5·7	18·6

NPU by carcass required thirty-two animals in eight cages and took 30 man-hours if N was determined in the carcasses, or 16 h if calculated from the ratio of N:water.

With all these drawbacks to PER, which have been discussed repeatedly over the years, it is surely time to abandon PER as a method, certainly as the standard method, of evaluating protein quality. It is more than half a century since Mitchell (1924) stated that simplicity is its only recommendation.
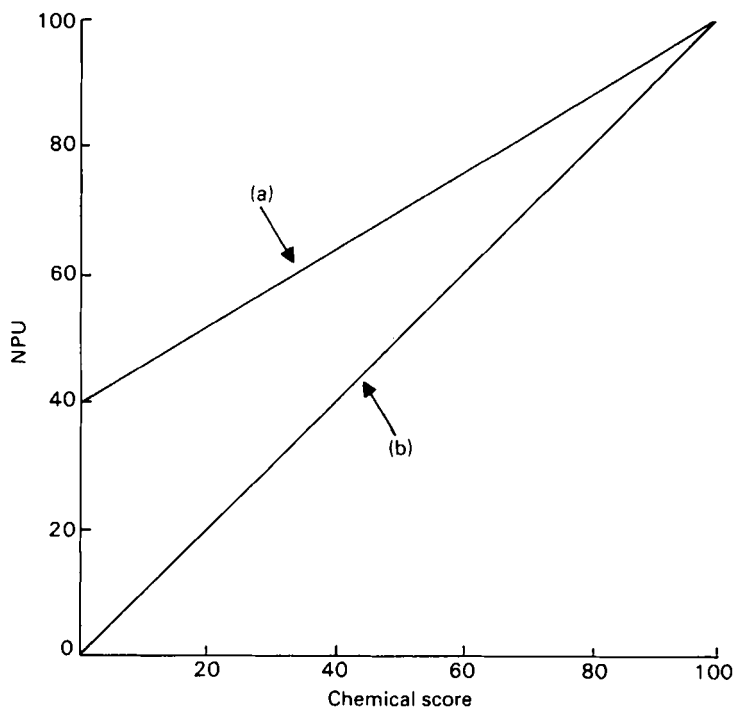

Fig. 1. Relationship between NPU and chemical score. (a) After Block & Mitchell (1946), (b) theoretical ideal relationship (after Bender, 1954; FAO, 1957).

Protein quality and AA composition

It was as recently as 1946 that Block & Mitchell (1946) showed a relationship between BV and AA composition. In view of the unreliability of AA estimations at that time and the use of results of measurements obtained in different laboratories from different samples, it is surprising that such a correlation could be shown (Fig. 1). The theoretical relation between NPU and chemical score (total lysine) is the straight line through zero. In practice, using AA mixtures of known composition, the relationships shown in Fig. 2 are obtained. AA mixtures completely lacking in lysine have been able to sustain life in rats for periods longer than 6 months (Bender, 1961) and the variable BV (20–40) found with lysine-free mixtures may be due to the varying ability to synthesise lysine or to the re-use of lysine. Khan & Eggum (1979) found that, when total lysine in a processed mixture fell by 75%, which should have reduced BV from 61 to 14, the BV was found to be 40. With a rice-based diet the fall in BV should have been from 71 to 33 but the BV was found to be 42. Had available lysine been measured the expected fall would probably have been much greater.

When there are eight (or ten) essential AA supplied by a protein it is not very helpful to provide a single figure for BV, however precise. What we need to know is
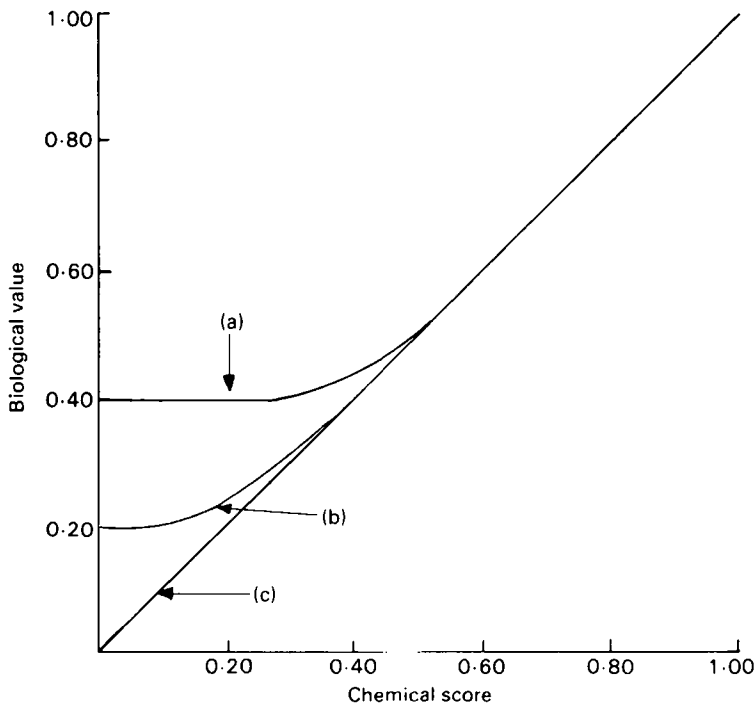

Fig. 2.   Relationship between BV and chemical score. Protein limited by (a) lys, (b) trp, thr, his, phe, leu and ile, (c) val and S-AA.

how much of each of the essential AA is present in the food in an available form and thence predict its value under a variety of conditions.

The objection to chemical analysis is that the AA may not be completely available. In many foods, however, particularly those based on cereals, the reduced availability applies only to lysine (so far as is known) since the addition of lysine to an overheated product restores the value. In the classical experiment of Block *et al.* (1946) the PER of a mixture was reduced by heat treatment from 3·6 to 0·8 and restored to 3·6 on adding lysine. (There may have been some reduction in surplus amount of S-AA but there is no evidence available and this was obviously not limiting at any stage.) In such foods the chemical method of Hurrell & Carpenter (1975, 1979) for measuring available lysine provides a great deal of the information required. Moreover, Almas & Khan (1981) found a correlation coefficient of 0·99 between chemically available lysine and chemical score (total lysine) implying that all the lysine was available in four mixtures of bread with legumes and one of rice with legumes.

In some foods processing may reduce the amount of S-AA or render them less available. We showed that fish meals ranging in NPU from 0·16 to 0·75 were all restored to 0·75 by the addition of methionine. (Again, there may have been a surplus of lysine which may have suffered damage but it was not limiting at any stage.)

It appears likely that human diets are limited by S-AA, lysine or threonine, so possibly only these three need to be examined. Available lysine may serve as an index of reduced availability of the other AA but not necessarily. There are no chemical methods validated for available S-AA or threonine but it is possible to carry out a 'biological analysis' as a final support for AA content as shown in Table 4.

'True' value

It might appear likely that if only the PQE could be carried out on human subjects this would provide the correct result. Such a procedure, however, combines both the physiological variables of the subjects with the composition of the food protein, and still depends only on the limiting AA. Calloway & Margen (1971), for example, found that BV of egg protein in human assays varied between

Table 4. *Biological analysis of available amino acids*

Food	BV	Conclusion
Bread	46	The limiting AA was present at 46% of target[*]
Bread + lysine	57	The first limiting AA was lysine; the second was present at 57% of target
Bread + lysine + threonine	70	The second limiting AA was threonine; the third was present at 70% of target
Bread + lysine + threonine + methionine	79	The third limiting AA was methionine; the fourth was present at 79% of target

[*]The target is the amount of each essential AA required for maximum NPU (Bender, 1958).

0·45 and 0·85, and the minimum amount of egg for zero N balance ranged between 3·9 and 6·8 g N/d.

Such findings are usual in all nutritional work. Layrisse & Martinez-Torres (1971), for example, found that in a group of only twenty-eight subjects the proportion of iron absorbed from soya ranged from 0·002 to 0·422. Whatever value is selected it is a combination of the chemical form of the iron, the presence of other dietary factors, and the physiological state of the subjects (Lock & Bender, 1980). The same may be true for proteins. Certainly rat assays are influenced by the type of carbohydrate, frequency of feeding, previous dietary state, etc. So it might be of greater value to know the available essential AA content of a food rather than to have, even were it possible, a BV, PER or any PQE carried out directly on man.

Conclusion

Enormous effort has been devoted over many years, firstly, to attempting to standardize methodology in the hope of detecting small differences in protein quality and, secondly, to finding quicker methods. Most of the reports in the literature refer to single protein sources and there are relatively few reports of the quality of the protein of complete diets and of the effects of processing and cooking mixtures of foods.

If the figures given in the FAO report (1973) are accepted, then the BV of the largely cereal diets of the developing countries is 0·7 (or rarely 0·6). It could be raised to 0·8 (that of western countries) by the plentiful addition of valuable protein foods such as meat and milk. Consequently, the protein quality of the diet of western countries appears to be of little importance. Currently there is concern that the replacement of meat by vegetable protein products might possibly lead to deterioration of protein quality in the diet of some individuals, and that the replacement of meat in manufactured products by collagen could similarly lead to a deterioration. If the difference between the protein quality of the diets of developing and developed countries is only between a BV of 0·7 and 0·8 then these concerns seem to be misplaced.

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