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A New Rapid Enzyme Digestion Method for Predicting *in Vitro* Protein Quality (PDD Index)

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ABSTRACT

The officially accepted Protein Efficiency Ratio (PER) assay has limited applicability as a quality control and process monitoring assay due to the 28 days required to complete the assay. The ultimate measure of protein nutritional quality is the human bio-assay; however, this takes from 35–45 days to complete and is very expensive compared to the PER assay.

PER is recognised by many nutritional biochemists as only an interim measure of human protein quality. The need for a reliable, timely, inexpensive rapid protein quality assay has become more evident in recent years with many rapid methods, including the Pepsin Digest Residue (PDR), Pepsin Pancreatin Digest (PPD), Pepsin Pancreatin Digest Dialysate (PPDD) and the Calculated Protein Efficiency Ratio (C-PER) methods, appearing in the research literature over the past 20 years. While each assay has notable merits, inherent limitations associated with each prevent use on an industrial basis. We now report a rapid assay, the Pepsin Digest Dialysate (PDD) Index, that can be used to determine protein quality over a wide range of protein sources currently available in human foods and which is acceptable for use in the food and nutritional product industries and for the Food and Drug Administration.

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INTRODUCTION

The human requirements for the 9 or 10 essential amino acids have been estimated (WHO, 1973) and these are provided, except *in utero*, largely in the form of intact proteins which require digestion to release their component amino acids and small peptides. For a normal adult, nitrogen excreted is equal to nitrogen intake. If the intake is altered then over a few days a new nitrogen balance is obtained either at a higher or lower level. There exists a lower limit of nitrogen intake below which nitrogen balance cannot be reached. Then the human body has a negative balance, where nitrogen excretion is greater than nitrogen intake. The lowest limit at which correct balance is possible is called the 'nitrogen balance equilibrium' or 'balance minimum'. This balance minimum is different for each protein; typical values (in g/day for a normal adult) being lactalbumin, 14; egg white, 17; and milk, 28.

Proteins vary from the ideal proportions in their content of constituent amino acids. Gelatin, for example, is completely devoid of one essential amino acid (tryptophan) and cannot achieve balance even if fed in very large quantities. It does not matter how complete a diet is, if one or more of the essential amino acids are missing, nitrogen equilibrium will not be maintained and the missing essential amino acids will be removed from the existing body mass and a wasting effect will be seen (Table 1).

Long before the isolation and characterisation of individual amino acids had been achieved it was recognised that proteins such as gelatin and plant proteins were inferior to other animal proteins. This early knowledge led to the concept of protein quality and thus methods concerned with the

TABLE 1
Limiting Essential Amino Acids in Animal and Plant Proteins

Protein	*First limiting amino acid*	*Other limiting amino acids*	*Biological value*[a]
Whole egg	None	None	100
Beef	Met, Cys	Phe, Val	76
Gelatin	Trp	Met, Cys, + others	25
Cows milk,	Arg	Met, Cys, Phe	90
Casein	Met, Cys	Arg	73
Lactalbumin	Arg	Phe	84
Whole corn	Lys	Trp	60
White flour	Lys	Trp, Val, Thr	52
Soya bean flour	Met, Cys	Val	75
Peanut	Met, Cys	Lys, Trp, Ile, Thr	58

[a] Percentage of absorbed nitrogen that is retained in the body for maintenance and/or growth.

TABLE 2
Essential Amino Acid Requirements and Deficiencies for Normal Human Adults

Amino acid	*Adult daily minimum requirement (mg)*	*Deficiency symptoms (man)*	*mg/100 g protein*			
			Egg	*Milk*	*Beef*	*Corn*
Arg	—	Retards spermatogenesis	700	122	1 220	380
His	—	—	240	72	620	200
Thr	500	Liver fat accumulation	560	152	845	300
Val	800	Negative nitrogen balance	790	233	975	425
Leu	1 100	Lack of growth and loss of weight	1 015	398	1 480	1 200
Ile	700	Low haemoglobin content	700	221	980	510
Lys	800	Nausea, dizziness, anemia	690	243	1 630	180
Met	1 100	Reduced growth	360	93	515	250
Phe	1 100	Reduced growth	640	181	740	400
Trp	250	Reduced growth and negative nitrogen balance	130	46	300	48

utilisation of protein nitrogen were developed. Many researchers during the last 75 years or so have been engaged in the sometimes frustrating problem of evaluating protein quality using a variety of methods (Satterlee *et al.*, 1977).

The two major purposes of protein quality evaluation are first to rank proteins according to their nutritive value and, second, to assess the efficiency of proteins in meeting human requirements for amino acids (Table 2). Despite studies on children and adults, over recent years, which have confirmed the effects on nitrogen balance of improving protein quality under appropriate test conditions, it is surprising to hear some nutritionists saying that protein quality does not matter in adults. Whilst it may not matter if you are in good health, it does matter if you are poor, sick, elderly or even pregnant or lactating. For children in a period of growth spurt or catch-up growth, protein quality can be even more important. A discussion into the methods, ethics and cost of human assays cannot be undertaken here, but Table 3 summarises some methods used in human protein quality assay along with the costs compared with chemical and microbiological methods.

In theory, we do not need a particularly complicated method for chemically assessing protein quality. All that is needed is a satisfactory method for measuring available amino acids and an accurate knowledge of human requirements for each essential amino acid (as shown in Table 2).

This approach has been used to evaluate food proteins by using the so called 'chemical score method' (Mitchell & Block, 1946). Until recently we were not in a position to rely on this method completely, because no measure of digestibility was taken into account and certain amino acids such as lysine, threonine and serine may not be fully available. In addition, human requirements for proteins vary considerably. Therefore, a biological method has always been used for measuring the amount of essential amino acids (EAA) in the greatest deficit relative to requirements.

TABLE 3
Comparison of the Methods Available for Assessing Protein Quality

Method	*Reference*	*Time to complete (days)*	*Approx. cost per assay (US$)*[a]
Animal and human methods			
Nitrogen balance studies (long term)	Young *et al.* (1977)	35–60	30000
Nitrogen balance studies (short term)	Lachance *et al.* (1979)	5–10	2000–5000
Rat growth studies			
Protein efficiency ratio (PER)	AOAC (1975)	28	350–600
Relative protein value (RPV)	Samonds & Hegsted (1977)	21	700–1000
Net protein utilisation (NPU)	Miller & Bender (1955)	10	200–400
Normalised protein ratio (NPR)	Bender & Doell (1957)	10	240–500
Enzymatic and bioassays			
Transaminase	Litwack *et al.*, (1952)	5	120–250
Tetrahymena	Kidder & Dewey (1951)	41	50–200
Chemical methods			
Pepsin pancreatin digest dialysate (PPDD)	Mauron (1955, 1973)	2–4	250–300
Pepsin digest residue (PDR)	Sheffner *et al.* (1956)	2–4	250–300
Calculated protein efficiency ratio (C-PER)	Satterlee *et al.* (1977)	2	200–300
Available lysine	Carpenter (1960)	1	100–200
Dye binding	Hurrel *et al.* (1979)	1	100–200
Pepsin pancreatin digest (PPD)	Akeson & Stalmann (1964)	1	100–200
Pepsin digest dialysate (PDD)	This paper	<2	150–200
Essential amino acid (EAA)	Oser (1959)	1	100–150
Chemical score	Bender (1961)	1	100–150

[a] Based on 1987 figures.

In view of the improved analytical equipment currently available for amino acid analysis, we can now dispense with bio-assays and use methods based on chemical score or EAA index. We report herein a new method, which covers the needs of the food and nutritional product industries, for the estimation of protein quality of a wide range of protein samples and which is relatively cheap, simple to perform and applicable to a wide range of food products including complex processed foods.

MATERIALS AND METHODS

Protein sources and foods tested

Standard protein samples purchased from Sigma Chemical Company, consisting of soya flour, gelatin BP, gluten, casein and whole egg powder were selected for this investigation. The commercial samples comprised several types of a fortified milk (normal cows milk with added carbohydrates, proteins and vitamins) for enteral hospital nutrition.

Enzyme used for *in vitro* digestion

Pepsin (EC 3.4.23.1), the enzyme used for *in vitro* digestion, was purchased from Sigma Chemical Company—(Number P7000, 1:10 000, 525 Units per mg of solid; one unit was defined as an increase in absorbance at 280 nm of 0·001 per minute at pH 2·0 at 37°C as trichloroacetic acid-soluble products).

Protein contents

The Kjeldahl method was used to determine total protein content of each protein, using copper or selenium as the digestion catalyst.

Reagents

The other reagents were of AnalaR (AR) or General Purpose Reagent (GPR) quality.

Preparation of samples for dialysis and digestion

For solid samples a known weight of sample (equivalent to 1·5 g protein calculated from the Kjeldahl protein value) was suspended in sodium chloride solution (1.5% in hydrochloric acid, 0·1M total volume 50 ml). Solid enzyme (37·5 mg) was added with thorough mixing and the solution transferred to presoaked dialysis tubing (Visking size 2) leaving a 2 cm air gap to aid agitation. The sealed dialysis bag was transferred to a 500 ml Schott bottle containing sodium chloride solution (1·5% in hydrochloric acid, 0·1M, 500 ml). The bottle was transferred to a shaking incubator at 37°C for 24 h (see Fig. 1). On completion of the incubation an aliquot (20 ml) of the dialysate was stored at −20°C until analysis for amino acid composition. Control solutions containing sample and no enzyme and enzyme but no sample were similarly treated.

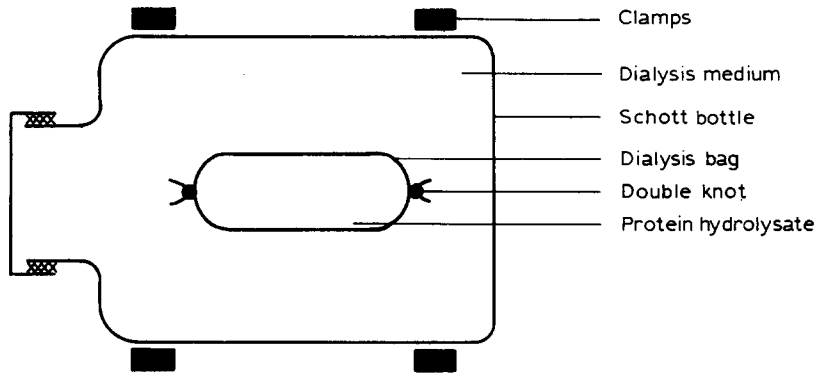

Fig. 1. Schematic representation of the shaking incubator used for the enzymatic digestion of food proteins.

For liquid samples a similar procedure was employed except that a volume of sample equivalent to 1·5 g protein was diluted to 50 ml with sodium chloride solution (as above).

Amino acid analysis

Amino acid analyses were performed on dialysate samples after hydrolysis by acid (6M HCl at 110°C for 24 h) or alkali (4M $Ba(OH)_2$ at 110°C for 16 h) as appropriate using a fully automated Locarte amino acid analyser equipped with a ninhydrin detection system. The results are shown in Tables 4 and 5.

Calculation of the pepsin digest dialysate (PDD) index

The method used for calculation of protein quality was adapted from that of Sheffner (1967) and based on the concentration of nine amino acids in the dialysate; namely, Thr, Val, Met, Ile, Leu, Phe, His, Lys and Arg. The inclusion of Arg and His in the calculation was to allow the index to be relevant to young and growing children and animals.

Calculation of the weight of each amino acid (in mg) in the dialysate was made from the amino acid analysis data as follows:

$$\text{Weight of amino acid in dialysate} = \left(\begin{matrix}\text{Concentration}\\ \text{of amino acid}\\ \text{in dialysate}\end{matrix}\right) \times \left(\begin{matrix}\text{Volume}\\ \text{of}\\ \text{dialysate}\end{matrix}\right) \times \left(\begin{matrix}\text{Molecular}\\ \text{weight of}\\ \text{amino acid}\end{matrix}\right) \times \left(\begin{matrix}\text{Normaliza-}\\ \text{tion}\\ \text{factor}\end{matrix}\right)$$

where the normalization factor is that factor required to give 1 g of protein equivalent.

The quantity of each amino acid remaining in the residue was then calculated by difference between the content of the individual nine amino

TABLE 4
Enzymatic Digestion and Dialysis of Various Food Proteins

Amino acid	*Amino acid content (nmol released per ml in 24 h)*											
	Whole egg		*Casein*		*Soya*		*Gelatin*		*Lactalbumin*		Gluten	
	Expt	*Control*	*Expt*	*Control*	*Expt*	*Control*	*Expt*	*Control*	*Expt*	*Control*	*Expt*	*Control*
Thr	540	22	250	0	260	4	32	8	650	0	220	8
Val	850	21	380	0	360	4	14	10	730	22	370	8
Met	450	0	75	0	71	0	10	0	240	0	120	0
Ile	640	15	160	0	320	2	23	7	570	0	380	5
Leu	1 040	20	860	0	600	2	75	9	1 450	35	560	10
Phe	530	0	380	0	320	1	62	7	360	0	260	8
His	190	0	100	0	140	10	21	14	200	0	140	19
Lys	570	8	250	0	510	0	48	0	760	0	100	0
Arg	530	0	170	0	450	27	66	13	230	0	180	8
Cys	25	0	32	0	29	0	0	0	47	0	14	0
Trp	150	—	80	—	—	—	—	—	—	—	—	—

Expt = Experiment: Conditions 500 ml dialysate pH 1·5 at 37°C, 24 h incubation in pepsin. Continuously agitated in a shaking incubator.
Control: as above but with no enzyme.
—: Not determined.

TABLE 5
Enzymatic Digestion and Dialysis of Various Milk-Based Enteral Products

Amino acid	*Amino acid content (nmol released per ml in 24 h)*									
	Product A		*Product B*		*Product C*		*Product D*		*Product E*	
	Expt	*Control*	*Expt*	*Control*	*Expt*	*Control*	*Expt*	*Control*	*Expt*	*Control*
Thr	800	80	410	7	420	15	430	22	290	0
Val	920	130	530	0	650	19	630	21	360	0
Met	270	40	150	0	160	7	140	0	140	0
Ile	660	110	360	10	360	25	440	15	220	0
Leu	1970	220	970	15	1080	40	1050	20	670	0
Phe	750	84	330	0	540	14	530	0	250	0
His	260	140	150	0	190	0	190	0	130	0
Lys	760	120	460	15	400	0	440	8	360	0
Arg	450	130	180	0	310	0	410	0	93	0
Cys	0	0	0	0	0	0	0	0	0	0

acids present in the total sample (see Table 6) and that calculated for the dialysate.

The relative amount of each amino acid (as a percentage of the sum of the nine essential amino acids) in each fraction was then calculated to give the so-called percentage patterns. These values were then expressed relative to the corresponding percentage patterns for the egg reference protein using

TABLE 6
Comparison of the Standard Food Proteins used in the PDD Experiments

Amino acid	*Molecular weight (g/mol)*	*Amino acid content (% w/w)*					
		Casein	*Whole egg*	*Soya isolate*	*Lactalbumin*	*Gluten*	*Gelatin*
Asp	133·10	5·40	4·17	4·4	6·91	2·21	5·90
Thr	119·10	3·58	2·09	1·6	3·20	1·81	1·89
Ser	105·09	4·58	3·12	2·1	2·90	3·00	3·71
Glu	147·13	15·90	5·19	7·2	11·70	24·80	10·09
Pro	115·13	8·31	1·29	2·2	3·50	8·50	26·68
Gly	75·07	1·30	1·51	1·4	1·20	1·90	24·15
Ala	89·09	2·32	2·06	1·6	3·30	1·70	9·84
Cys	121·16	0·31	1·02	0·4	1·90	1·50	Trace
Val	117·15	5·16	2·73	1·7	3·60	2·60	2·20
Met	149·21	2·48	1·48	0·4	1·80	1·20	0·79
Ile	131·18	4·15	2·33	1·8	3·60	2·50	1·39
Leu	131·18	7·29	3·80	2·9	8·60	4·30	2·95
Tyr	181·19	4·68	2·35	1·3	3·20	2·50	0·30
Phe	165·19	4·11	2·30	1·9	2·80	3·70	2·10
His	155·16	2·52	1·19	1·1	1·70	1·40	6·91
Lys	146·19	6·25	3·17	2·3	6·00	1·20	3·99
Arg	174·20	3·08	2·80	2·8	2·10	2·50	7·81
Trp	204·23	2·00	3·60	0	2·00	0·90	0
Total% w/w	—	81·4	42·6	37·1	71·4	73·98	95·0

TABLE 7
An Example of the PDD Index Calculation for Casein

Amino acid	*Casein*			*Whole egg*		
	Total	*Digest*	*Residue*	*Total*	*Digest*	*Residue*
Thr	44·0	28·3	15·7	49·1	20·0	29·1
Val	63·4	43·5	19·9	64·1	32·4	31·7
Met	30·5	17·3	13·2	34·7	18·2	16·5
Ile	51·0	28·0	23·0	54·6	22·8	31·8
Leu	89·6	72·9	16·7	89·2	44·2	45·0
Phe	50·5	39·3	11·2	54·0	27·0	27·0
His	32·0	13·3	17·7	27·9	7·0	20·9
Lys	76·8	39·2	37·6	74·4	25·4	49·0
Arg	37·8	30·2	17·6	65·7	21·1	44·6
Total	474·6	302·0	172·6	513·7	218·1	295·6
	d	*e*	*f*	*a*	*b*	*c*

Amino acid	*Pepsin digest*				*Residue*			
	Casein % (*1*)	*Egg %* (*2*)	*Egg ratio*	*Log (Egg ratio)*	*Casein %* (*5*)	*Egg %* (*6*)	*Egg ratio*	*Log (Egg ratio)*
Thr	9·4	9·2	100·0	2·000 0	9·1	9·8	92·9	1·967 8
Val	14·4	14·9	96·7	1·985 2	11·5	10·7	100·0	2·000 0
Met	5·7	8·3	68·7	1·836 9	7·6	5·6	100·0	2·000 0
Ile	9·3	10·5	88·6	1·947 4	13·3	10·8	100·0	2·000 0
Leu	24·1	20·3	100·0	2·000 0	9·7	15·2	63·8	1·804 8
Phe	13·0	12·4	100·0	2·000 0	6·5	9·1	71·4	1·853 6
His	4·4	3·2	100·0	2·000 0	10·3	7·1	100·0	2·000 0
Lys	13·0	11·7	100·0	2·000 0	21·8	16·6	100·0	2·000 0
Arg	6·7	9·7	69·0	1·828 8	10·2	15·1	67·5	1·829 3
Total log (Egg ratio)				17·608 3				17·455 5
Average of log (Egg ratios)				1·956 5				1·939 5
Geometric mean				90·50				86·99
Corrected	$90{\cdot}50 \times \frac{e}{b} = 90{\cdot}50 \times \frac{302{\cdot}0}{218{\cdot}1} = 125{\cdot}3$				$86{\cdot}99 \times \frac{f}{c} = 86{\cdot}99 \times \frac{172{\cdot}6}{295{\cdot}6} = 50{\cdot}8$			

$$\text{PDR Index} = \left(\frac{100b}{a} \times \log 125{\cdot}3\right) + \left(\frac{100c}{a} \times \log 50{\cdot}8\right)$$

$$= \text{antilog}\,(0{\cdot}425 \times \log 125{\cdot}3 + 0{\cdot}575 \times \log 50{\cdot}8)$$
$$= \text{antilog}\,(1{\cdot}8728)$$
$$= 75{\cdot}62$$

the egg ratio method (Mitchell & Block, 1946). In order to avoid the use of negative logarithms and percentage concentrations in excess of those present in the standard protein, any egg ratio values greater than 100% were assumed to be 100% (Oser, 1951).

The geometric mean of the egg ratio values for both the digest and residue fractions was then calculated as the antilog of the mean of the sum of the log (egg ratio) values. In order to indicate how this quantitative parameter for each fraction contributes to the total value, two adjustments were required.

(a) Correction for proteolysis. A factor, obtained by dividing the total amount of the nine essential amino acids in the digest of the test protein sample by the same value obtained for the reference egg protein, was used to correct the geometric mean of the total egg ratio of the pepsin digest fraction. A similar factor for the residue fraction was used to correct the residue geometric mean.

(b) Correction for the qualitative importance of the two fractions. The corrected geometric mean values were corrected by the ratio of the total amount of the nine essential amino acids in the digest and residue fractions relative to the total amount, respectively.

Following these corrections the PDD index of the whole protein was calculated as the geometric mean of these corrected values. For clarity a worked example is shown below the specimen data sheet used for the computations in Table 7. The calculation of PDD index was performed using a Commodore PET micro-computer.*

Correlation between *in vitro* and *in vivo* parameters

In vivo rat biological values were obtained from the literature (Satterlee *et al.*, 1977; Hsu *et al.*, 1977 and Jewell *et al.*, 1980) and compared with the PDD index values obtained in this study.

RESULTS AND DISCUSSION

In recent years the FDA, either in regulations or proposed regulations dealing with food labelling, has included sections which affect the labelling of the protein contribution of a product depending upon its quality as measured by PER (Protein Efficiency Ratio). For example the FDA has stated:-

> The US RDA (Required Daily Average) of protein in a food product is 45 g if the Protein Efficiency Ratio (PER) of the total protein in the

* Details of the computer program can be obtained from Dr. J. F. Kennedy.

> product is equal to or greater than that of casein, and 65 g if the PER of the total protein in the product is less than that of casein.

Furthermore, the FDA has defined quite precisely the total protein content of a food:-

> Total protein with a PER of less than 20% of the PER of casein may not be stated on the label in terms of percentage US RDA, and the statement of protein content in grams *per* serving shall be modified by the statement 'Not a significant source of protein' immediately adjacent to the protein content statement regardless of the actual amount of protein present.

To date, the PER method of assessing protein quality is the only official recognised method (AOAC, 1975). This is a biological method and has been heavily criticised by several nutritional biochemists:-

(a) As a research tool it is known to underestimate the quality of lower quality proteins.
(b) It is non-linear in its estimation of protein quality.
(c) It is both too expensive and time consuming to perform.
(d) It yields non-reproducible data, primarily because each laboratory which runs the bioassay does so with slight procedural modifications.

The FDA have agreed that the rat PER method is inadequate (Adkins, 1977) and would be amenable to change the official assay requirement away from the PER when the scientific community agrees upon a more suitable assay for measuring protein quality. Table 8 gives us some indication of what the FDA precisely requires. The most obvious point to note is that a chemical method is required, the reason for which is fairly obvious, as shown by Table 3.

Although the amino acid composition of a protein is fundamentally

TABLE 8

List of Essential Features of a New Official Method of Protein Quality Assay According to 1977 FDA Guidelines

1. Accuracy, reproducibility.
2. Time to complete the assay must not exceed 2 weeks (preferably less than 2 days).
3. The assay should not use human subjects, but can be verified through the use of clinical studies.
4. The method must include an assessment of digestibility comparable to that which would be found in human digestion.
5. The assay would use a representative aliquot of the sample without extensive preparation.
6. The method should have endorsement by the AOAC.
7. The costs of the assay should not exceed 200 dollars (1977 prices).

TABLE 9

Structural Features of a Protein Food Product which may Affect Protein Quality

A. *Structure of the protein*
- i. Primary Structure
  - (a) Amino acid composition.
  - (b) Total and relative amounts of amino acids (may be modified by processing and storage).
- ii. Secondary Structure
  Sequence of amino acid residues.
- iii. Tertiary Structure
  Non-bonded interaction between side chains and/or between side chains and amide bonds to give alpha-helix, beta-pleated sheet or other structure (may be modified by processing and storage).
- iv. Quaternary Structure
  Intra-molecular bonding (may be modified by processing and storage).

B. *Matrix of the protein*
- i. Cell walls.
- ii. Inhibitors.
- iii. Inter-molecular bonding (may be modified by processing and storage).

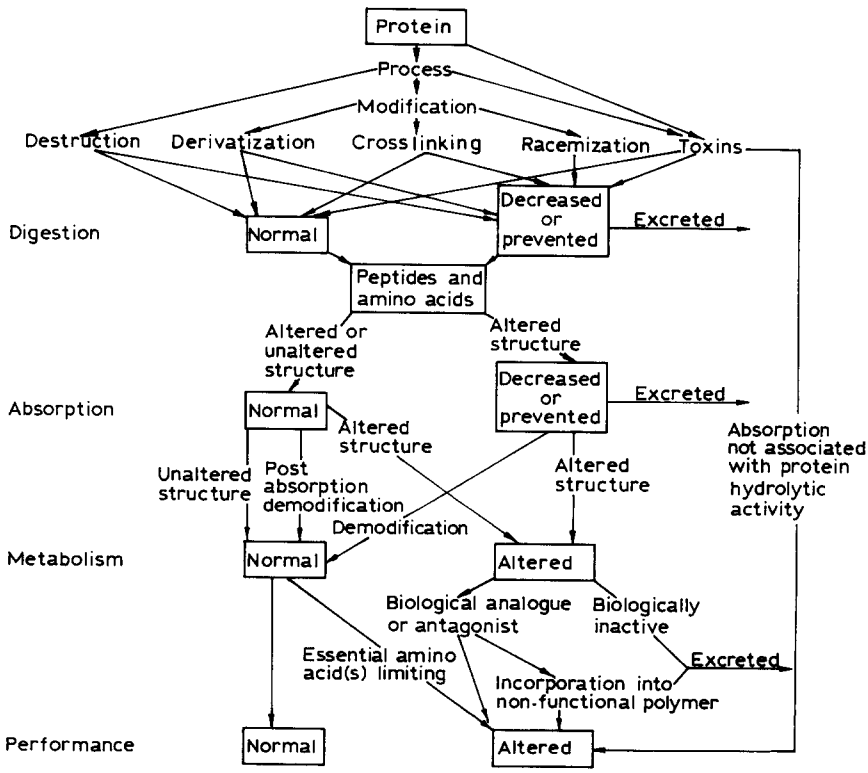

Fig. 2. Possible consequences of metabolism of modified proteins.

related to its nutritional quality, protein quality cannot be reliably predicted from amino acid composition alone. Obviously, bioavailability is one factor which must be considered. Digestibility and absorbability (bio-availability) may be affected by characteristics of the food proteins themselves, including protein conformation, intermolecular bonding, and modification of the amino acids by heat-processing and storage histories (see Table 9 and Fig. 2). Bioavailability may also be affected by the matrix of the protein in the foodstuff, such as containment of the protein inside indigestible cell walls, presence of inhibitors or toxic factors. Thus an *in vitro* assay combining amino acid analysis with digestibility is a most obvious method to develop.

Several approaches have been made to develop a suitable method which encompasses both protein quality and digestibility. Four methods will be discussed briefly.

(1) Pepsin digest residue (PDR Index)

Sheffner *et al.* (1956) determined the amino acid composition of food proteins after their digestion with pepsin. Using these values to calculate EAA indices, results were found to be in agreement with Net Protein Utilization (NPU) values. A factor of digestibility can be calculated for each amino acid by finding the difference between amino acids determined from acid hydrolysis and those determined from pepsin hydrolysis.

Sheffner's method is a very simple open digestion apparatus which uses one enzyme (pepsin), this tends to make the test relatively rapid. The reactions are stopped by acid/tungstate precipitation followed by centrifugation. The supernatant is then analysed for amino acids, but the supernatant prepared in this way tends to clog the analytical column.

(2) Pepsin pancreatin digest (PPD Index)

This is a modification of the PDR index of Sheffner, using both pepsin and pancreatin to hydrolyse protein to amino acid. The PPD index is calculated in the same manner as the PDR index. The results are not noticeably improved by using two digestive enzymes as opposed to one, the method is longer because two enzymes are used. Automated amino acid analysis was used for the first time in this method.

(3) Pepsin pancreatin digest dialysate (PPDD Index)

This is a modification of the PPD index (Mauron *et al.*, 1955, Mauron, 1973). The protein digest is dialysed during the pancreatin stage and aliquots of the dialysate are removed at regular intervals. The calculation of the

PPDD index is the same as that used for the PDR index. The difference between the PPD and the PPDD indices lies in the fact that with the PPDD index, amino acids are weighted according to the speed of enzymatic release, and the use of a dialysis membrane simulated the gut more realistically. The correlation with biological value was more linear than previous methods. Unfortunately this method required complex equipment, difficult manipulations, pooling of samples, and was, above all, labour intensive and time-consuming.

(4) Calculated protein efficiency ratio (C-PER Index)

This method, devised by Satterlee (1977) uses a different approach from those discussed above in that digestibility is measured by a drop in pH of a protein mixture after hydrolysis with a cocktail of four endo- and exo-acting proteases. The pH drop is inversely related to rat *in vivo* digestibility and is used to calculate the protein efficiency ratio by combination of data of the complete amino acid profile of the protein samples. Whilst the method is undoubtedly fast it suffers from a number of major shortcomings:

(a) the accuracy with which the pH drop can be measured is low.
(b) correlation with *in vivo* data is poor.
(c) complex statistical data manipulation is required.

Despite these shortcomings which affect the accuracy of the method, it has been given first official action approval by the AOAC (Satterlee, 1984).

The pepsin digest dialysate (PDD) index

In order to overcome many of the problems of the above-mentioned methods we have devised a method which:-

(a) uses simple apparatus.
(b) uses a single enzyme.
(c) employs a dialysis process which is easy to handle.
(d) utilises conventional automated amino acid analysers.
(e) can be calculated manually or with the aid of a simple microcomputer.
(f) can be performed in less than two days.
(g) is relatively cheap to perform.

The amino acid profiles of the six standard food proteins used in this work are shown in Table 6. The six proteins chosen (egg, lactalbumin, casein, soya, gluten and gelatin) gave a good spread of biological values with the range 25 to 100.

The essential amino acid release profiles of the pepsin dialysate digests of the six standard food proteins after 24 h incubation with and without

TABLE 10
Reproducibility of Six Egg Protein Control Dialysates (all amino acids)

Amino acid	*Amino acid concentration (nmol/ml)*							
	Digest numbers							
	1	*2*	*3*	*4*	*5*	*6*	*Mean*	*SD*
Asp	930	670	920	860	790	1 150	886·56	160·8
Thr	540	360	500	470	430	660	493·33	102·3
Ser	1 000	660	980	860	770	1 090	893·33	160·2
Glu	1 410	910	1 270	1 130	1040	1 350	1 185·00	192·2
Pro	510	360	480	490	420	580	471·66	75·4
Gly	630	410	530	540	510	710	555·00	103·4
Ala	950	700	830	910	860	1 160	901·66	152·7
Cys	25	40	31	30	19	40	30·83	8·2
Val	850	570	730	750	690	960	758·33	134·2
Met	450	310	400	410	360	490	403·33	63·8
Ile	640	480	610	620	570	790	618·33	101·5
Leu	1 040	740	890	940	880	1 190	782·50	211·8
Tyr	340	270	420	360	330	460	363·33	67·7
Phe	530	410	560	560	510	690	543·33	90·7
His	190	130	170	180	170	280	186·67	50·0
Lys	570	400	500	510	480	830	548·33	148·5
Arg	530	330	470	440	470	590	471·67	87·7
Trp	150	60	130	90	70	0	100·00	38·7

pepsin at 37°C are shown in Table 4. The results clearly show that lactalbumin and casein give the greatest release after 24 h incubation and gelatin gives the poorest release. In fact gelatin is poorly digested and has a poor raw material amino acid sequence, as well as poor release. All the proteins investigated in Table 4 are at equiprotein levels so all data are directly comparable.

Similarly, Table 5 shows the essential amino acid release profiles of the five commercial milk products fortified with added carbohydrates, proteins and vitamins for nasogastric feeding. Again all are directly comparable as they are on an equiprotein basis. It seems that they all are very similar in their release rates after 24 h.

The reproducibility in terms of mean and standard deviation of some of the egg protein controls that were carried out shows that the results are satisfactory (see Table 10) for this experimental set up (coefficient of variation $\sim$15–20%) of variable proteins and enzyme reaction and amino acid analysis of a small volume and low concentration of amino acid. Similarly Table 11 shows duplicate experiments of the full amino acid release of one of the nutritional milk products. The results in these two experiments are very close and the reproducibility is very high.

TABLE 11
Duplicates of Digestion and Control of a Sample of a Commercial Milk Product Fortified with added Carbohydrates, Proteins and Vitamins

Amino acid	*Amino acid content (nmol/ml)*		
	1	*2*	*Control*[a]
Asp	680	640	26
Thr	420	390	22
Ser	550	530	17
Glu	1430	1350	58
Pro	1100	990	0
Gly	320	400	27
Ala	400	450	18
Cys	0	0	0
Val	650	620	18
Met	160	150	0
Ile	360	340	18
Leu	1080	1020	27
Tyr	500	490	45
Phe	540	510	25
His	190	180	0
Lys	400	370	15
Arg	310	260	0
Trp	90	80	0

[a] Control = sample plus buffer alone.

The calculated pepsin digest dialysate (PDD) index was plotted as a function of biological values obtained from the literature for the standard food proteins used (see Fig. 3). The linear regression equation found was $y = -5{\cdot}9 + 1{\cdot}0859x$, $r = 0{\cdot}9928$. The same figure shows chemical score, essential amino acid index, Mauron's PPDD index and Stalmann's PPD index also plotted as a function of biological value. The linear regression lines are as follows:-

	linear regression	r
PDD Index	$y = -5{\cdot}9 + 1{\cdot}0859x$	0·9928
Chemical Score	$y = -3{\cdot}71 + 0{\cdot}9930x$	0·9904
Essential Amino Acid Index	$y = 15{\cdot}92 + 0{\cdot}924x$	0·9652
PPD Index (Akeson and Stalmann)	$y = -13{\cdot}574 + 1{\cdot}1209x$	0·9962
PPDD Index (Mauron)	$y = -6{\cdot}865 + 1{\cdot}0165x$	0·9860

The reproducibility of the technique in examining four different types of enteral milk products is shown in Table 12. It is apparent that the inter-experiment variation is very satisfactory for all the products examined.

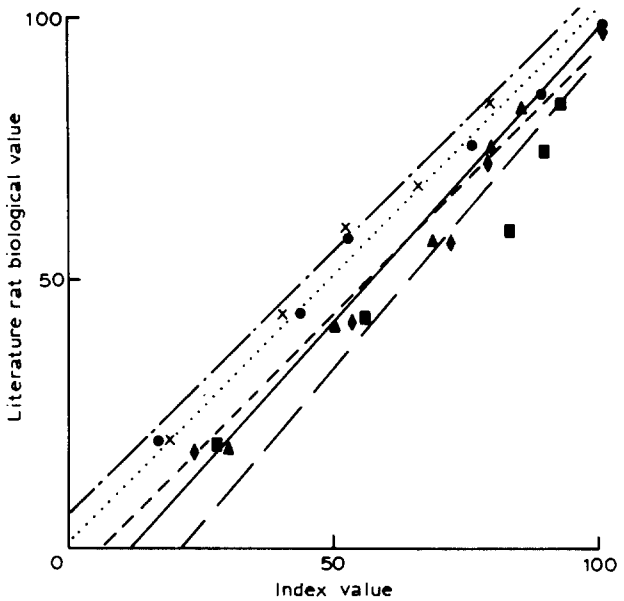

Fig. 3. Correlation between the rat biological value of proteins and the various *in vitro* indices devised (●·····● pepsin digest dialysate; —◆— —◆— pepsin pancreatin digest dialysate (Mauron, 1973); —▲—▲— pepsin pancreatin digest (Akeson & Stalmann 1964); —■— —■— essential amino acid (Oser, 1959); and —×— · —×— chemical score (Bender 1961)).

It is concluded therefore that the PDD Index method described in this paper provides a rapid estimate of the protein quality of a food (in this case enteral milk products) within 48–72 h. The method has advantages over the widely used C-PER method which has been described as crude and

TABLE 12
Reproducibility of the PDD Method using Milk Based Enteral Products

Product	*PDD Index*
Type 1	83·2
	83·0
Type 2	68·0
	67·9
	67·7
Type 3	75·0
	76·0
Type 4	83·6
	88·4
	86·2

inaccurate. This method also has advantages over the previously described digestion methods of Mauron and of Sheffner in that it:—

(a) uses a simpler and more easily handled apparatus.
(b) the stirring of the incubation mixture is more efficient.
(c) only one enzyme incubation temperature vessel and medium are required.
(d) is biologically explainable in that the factors used to predict the protein quality are its degree of digestibility as well as its essential amino acid profile.
(e) uses modern amino acid analysis.
(f) has good correlation with rat biological values.
(g) has been shown to be reproducible.
(h) has a computerised calculation.
(i) provides information as to why a protein has a high or low protein quality by indicating the degree of protein digestibility and by quantitating the degree to which each essential amino acid is limiting in the protein.

Whole egg was chosen as the standard and given a value of 100 by definition; thus, the PDDI method slightly over-estimated biological value by 1–2%. Many of the indices were calculated without tryptophan; however, Rose (1937 and 1938) showed that the minimal level of tryptophan required for growth was lower than for other essential amino acids, and it was therefore considered that good estimations can be made of the biological value without including the tryptophan correction. Thus, by eliminating the need for a separate alkali hydrolysis for tryptophan estimation, this reduced the time and expense of the assay.

The linear regression equations obtained for the most commonly used *in vitro* indices are shown on previous pages. It is obvious that the chemical score index of Mitchell & Block (1946) underestimated the biological value whereas the essential amino acid index of Oser (1951) overestimated the biological value. Similarly, the PDR method of Sheffner (1967) overestimates the biological value slightly.

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