2-Aminoethylphosphonic acid as an indicator of *Tetrahymena pyriformis* W growth in protein-quality evaluation assay

BY JULITA MACIEJEWICZ-RYŚ AND ANNA M. ANTONIEWICZ

Department of Animal Nutrition, Institute of Animal Husbandry, Skr. 161, 30–960 *Kraków, Poland*

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1. The concentration of 2-aminoethylphosphonic acid (AEP) in 96 h cultures of *Tetrahymena pyriformis* W was studied in order to apply it as an indicator in the assay of the relative nutritive value (RNV; protozoa population with test protein: protozoa population with whole-egg powder) of protein. Foodstuffs and food mixtures of different protein contents and qualities were used as test samples.

2. RNV values based on AEP determination (RNV_{AEP}) were compared with corresponding values calculated from protozoa counts (RNV_{pc}), as well as with biological value (BV) and net protein utilization (NPU) of the same proteins assayed on rats.

3. Both for foodstuffs and food mixtures highly significant correlations were found between RNV_{AEP} and RNV_{pc}, RNV_{AEP} and both BV and NPU, and RNV_{pc} and both BV and NPU.

4. AEP content in the protozoal suspension was preferred to cell count as a measure of growth response, since it took into account large differences in cell dimensions that were observed between cultures grown with different test proteins.

The method for the evaluation of protein quality using the protozoon *Tetrahymena pyriformis* W (Fernell & Rosen, 1956) as modified by Stott, Smith & Rosen (1963) involves the determination of the number of protozoa organisms after incubation for 4 d in a medium containing the protein to be evaluated. The total number of protozoa cannot be measured turbidimetrically because many foodstuffs are insoluble and coloured.

Total protozoa count, estimated using a haemocytometer, has often been made (Fernell & Rosen, 1956; Teunisson, 1961; Rosen, Stott & Smith, 1962; Stott *et al.* 1963; Baum & Haenel, 1965; Rølle & Eggum, 1971) but such a procedure is laborious and time-consuming (Teunisson, 1971; Shorrock & Ford, 1973). For many years, therefore, studies have been made of the potential use of other methods for protozoal population density determination.

Teunisson (1971) proposed a method for counting the cells electronically with a Coulter Counter, after separating *T. pyriformis* W cells from food particles. Attempts to apply acidity determination (Rockland & Dunn, 1949) or a colorimetric method (Anderson & Williams, 1951; Viswanatha & Liener, 1955; Bergner, Münchow & Koch, 1968) in the measurement of growth responses gave poor results.

Methods based on extinction measurements have been applied to alkaline extracts of soluble proteins (Vořišek & Leitgeb, 1973) and to high-protein meals after treatment with papain (Shorrock & Ford, 1973), but they are not widely applicable.

Shepherd, Taylor & Wilton (1975) suggested the estimation of tetrahymanol, a specific pentacyclic terpene synthesized by *T. pyriformis*, as an index of protozoal growth. Another compound, 2-aminoethylphosphonic acid (ciliatine; AEP) in *T. pyriformis* cells was detected by Kandatsu & Horiguchi (1962). Abou Akkada, Messmer, Fina & Bartley (1968), Ibrahim & Ingalls (1972) and Czerkawski (1974) suggested the use of AEP as an index of protozoal protein synthesis in the rumen. Because of the high content in *T. pyriformis* cells (Rosenberg, 1964) and its absence in natural foodstuffs (Abou Akkada *et al.* 1968), AEP content can serve as an index of protozoal growth in Tetrahymena assays for protein quality.

The present investigation was undertaken to compare relative nutritive values (RNV) for

foodstuffs and food mixtures, estimated both from protozoa counts in a haemocytometer and from AEP content. The results were also compared with biological value (BV) and net protein utilization (NPU) results determined with rats.

MATERIALS AND METHODS

Materials

The foodstuffs tested were chosen to illustrate differences in quality within and between different protein sources. The samples comprised: fish meals (FM26, FM33, FM45, FM58), blood meals (BM22, BM31, BM40, BM61), meat-and-bone meals (MBM34, MBM47, MBM59), extracted soya-bean meals (SB35, SB38, SB57), groundnut meals (GN32, GN36, GN62), lupin (*Lupinus luteus*) meal (L39, L56), field peas (*Pisum sativum*) (FP37, FP55), field beans (*Vicia faba*) (FB20, FB51), barleys (B21, B41), yeasts (Y42, Y63) and rape (*Brassica napus*) (R43).

Food mixtures for fattening pigs, made from components listed previously were also evaluated: high-protein concentrates M1, M10, M52, M64 were based on high-protein foodstuffs; high-protein mixtures M53, M54 contained lucerne(*Medicago sativa*)-protein concentrate ('Vepex', Budapest, Hungary) at 550 and 700 g/kg mixtures, respectively; concentrates M2, M65, M66 contained decreased proportions of protein of animal origin; M3, M4, M6, M11 were low-protein plant mixtures; these latter mixtures were also given supplemented with synthetic lysine (Lys) (M4+Lys, M6+Lys), synthetic methionine (Met) (M6+Met, M11+Met, M67) and both lysine and methionine (M6+Lys+Met, M11+Lys+Met).

Methods

RNV as a measure of protein quality was calculated as protozoal population in the test medium: protozoal population in an equinitrogenous medium containing whole-egg powder, the nutritive value of which was assumed to be 100. Protozoal populations were assessed directly by counting the numbers of organisms, and indirectly by AEP determination.

1. *RNV assay based on protozoa count* (RNV_{pc})

The assay procedure was that of Stott *et al.* (1963). The basal medium solution was prepared according to Baum & Haenel (1965). Since a previous study (Maciejewicz, 1972) showed that predigestion of the foodstuff with papain improved the agreement between RNV evaluated with *T. pyriformis* and BV of protein determined with rats, all foodstuffs and food mixtures were subjected to this process. Samples containing 50 mg protein-N were digested with 1 ml of a suspension of crude papain (Loba-Chemie, Wien-Fischamend, Austria) (40 mg/ml) according to the method described by Boyne, Price, Rosen & Stott (1967). Then the digest samples were brought to pH 8·2 and diluted to contain 0·5 mg N/ml. Portions (6 ml) of the diluted digests together with 2 ml basal medium (Stott *et al.* 1963) were sterilized at 121° for 10 min in 100 ml Ehrlenmeyer flasks. After cooling, 1 ml of vitamin solution (Baum & Haenel, 1965) and of glucose solution (150 g/l) were added under sterile conditions. All the flasks were then inoculated with 0·2 ml of a 3 d culture of *T. pyriformis* W and incubated for 96 h at 25°.

The protozoa count was made using a Fuchs–Rosenthal haemocytometer (Fein-Optik, Bad Blankfnburg, Germany) in samples prepared according to Stott *et al.* (1963). In the calculation of RNV a 'blank' correction was applied, obtained from cultures in which the papain represented the only source of N.

Measurement of protozoal dimensions. The measurements of length and width of protozoa cells were made using the micrometric eyepiece of a light-microscope. The dimensions of all

protozoa on an area of 1 mm^2 were measured. The measurements were made in triplicate and the mean results were calculated.

2. *RNV assay based on AEP determination (RNV_{AEP})*

The procedure used for AEP determination was that of Czerkawski (1974). Protozoal suspensions obtained from the same incubations as for the RNV_{pc} assay (each of 20 ml) were hydrolysed with the equal volume of 12 M-hydrochloric acid in sealed glass-tubes (30 × 100 mm) for 48 h at 110°. HCl was removed by evaporating to dryness three times, using a rotary evaporator (Büchi, Switzerland) at 60°. The residue was dissolved in 0·2 M-formic acid (5 ml). Portions (2 ml) were applied to a 8 × 120 mm column of Dowex 1X8 (200–400 mesh) (Fluka & Buchs, Switzerland) that had been previously equilibrated with 0·2 M-formic acid. AEP was eluted from the column with 30 ml 0·2 M-formic acid. The eluate was concentrated by evaporation at 60°. AEP was determined from its phosphorus content after hydrolysis (0·5 ml sample in test-tube) by ashing with 0·22 ml 37 M-sulphuric acid and 0·16 ml 12 M-perchloric acid. Phosphomolybdate blue colour was developed according to Fiske & Subbarow (1925), in the same test-tube in which the hydrolysis was done. For each sample the total AEP-P determined was corrected for any inorganic phosphate remaining after Dowex-column separation.

3. *BV assay with rats*

BV of proteins in foodstuffs and food mixtures was assayed using growing rats by a modified Thomas–Mitchell method as described by Eggum (1973).

RESULTS

Table 1 gives the comparison of RNV_{pc}, RNV_{AEP}, BV and NPU values for the protein in foodstuffs. For some foods the values obtained by the microbial method (RNV_{pc} and RNV_{AEP}) were in fair agreement with BV values of these proteins, estimated on rats. The proteins of fish meals and of soya-bean meals were the highest in quality and that of blood meals was the lowest, as evaluated by both rat and microbiological methods.

For some foodstuffs, however, there were marked discrepancies between RNV and BV. Thus RNV_{pc} and RNV_{AEP} were too high for meat and bone meals MBM47 and MBM59. Differences were also noted for rape R43 and barley B41, RNVs of which were lower than the respective BV and NPU values.

Within the groups of similar materials (fish meals and blood meals) higher values of BV corresponded with higher RNV (i.e. FM45 and FM58; BM22, BM61 and BM40). The same was not observed for lupins and field beans, where BV might have been affected by trypsin inhibitor action, which would not influence growth of *T. pyriformis* (Viswanatha & Liener, 1955).

A highly significant correlation (r 0·96, $P = 0{\cdot}01$) was found between RNV_{pc} and RNV_{AEP}. Highly significant correlations between both RNV_{pc} and RNV_{AEP} and BV (r 0·67, $P = 0{\cdot}01$; r 0·69, $P = 0{\cdot}01$ respectively) as well as between both RNV_{pc} and RNV_{AEP} and NPU (r 0·68, $P = 0{\cdot}01$; r 0·70, $P = 0{\cdot}01$ respectively) were observed. If the values for meat and bone meals were omitted from calculations, the correlation coefficients increased respectively to 0·98, 0·90, 0·89, 0·88 and 0·87, all at $P = 0{\cdot}01$.

Simple regression equations, calculated after excluding the values for meat and bone meals were: BV = 0·8415 RNV_{pc} + 8·0146; NPU = 0·8020 RNV_{pc} + 3·9980 and BV = 0·8864 RNV_{AEP} + 6·7353; NPU = 0·8817 RNV_{AEP} + 1·0770.

Table 1. *The comparison of the relative nutritive value* of proteins of different foodstuffs, as determined microbiologically using* Tetrahymena pyriformis *W* (RNV_{pc} *and* RNV_{AEP}) *and their biological value* (BV) *and net protein utilization* (NPU) *assayed on rats*

(Mean values for two determinations)

Sample	*T. pyriformis* assay		Rat assay	
	RNV_{pc}† (*a*)	RNV_{AEP}‡ (*b*)	BV (*c*)	NPU (*d*)
Whole-egg powder	100·0	100·0	98·9§	—
Fish meal				
FM26	71·5	72·2	70·0	61·8
FM33	54·6	58·3	—	—
FM45	76·5	72·1	67·9	63·8
FM58	69·4	68·0	64·4	62·0
Blood meal				
BM22	21·8	23·2	16·0	11·9
BM31	28·3	29·6	—	—
BM40	29·5	35·8	32·3	28·8
BM61	25·8	27·0	29·3	22·1
Meat-and-bone meal				
MBM34	43·4	48·5	—	—
MBM47	80·8	69·3	42·6	36·9
MBM59	65·4	63·6	37·9	32·8
Groundnut meal				
GN32	58·3	57·6	—	—
GN36	59·4	54·5	51·8	47·2
GN62	60·2	54·5	52·1	49·5
Soya-bean meal				
SB35	74·7	74·3	—	—
SB38	74·0	68·8	66·4	61·1
SB57	72·3	70·9	66·5	63·3
Lupin (*Lupinus luteus*)				
L39	49·9	47·2	52·5	48·2
L56	50·7	48·1	61·1	57·8
Field pea (*Pisum sativum*)				
FP37	46·0	53·4	47·2	41·5
FP55	54·5	50·5	58·3	50·5
Field bean (*Vicia faba*)				
FB20	44·9	43·0	41·2	32·7
FB51	45·3	47·0	46·3	42·1
Barley				
B21	53·0	50·7	48·8	40·8
B41	43·7	46·3	61·2	54·0
Yeast				
Y42	56·2	56·8	45·3	38·6
Y63	57·2	46·4	51·6	44·8
Rape (*Brassica napus*) ground				
R43	44·2	40·3	62·1	49·4

Correlation coefficients: *a,b*, 0·96; *a,c*, 0·67; *a,d*, 0·68; *b,c*, 0·69; *b,d*, 0·70; all at $P = 0·01$.

Correlation coefficients after omitting values for meat-and-bone meals: *a,b*, 0·98; *a,c*, 0·90; *a,d*, 0·89; *b,c*, 0·88; *b,d*, 0·87; all at $P = 0·01$.

* Protozoa population with test protein: protozoa population with whole-egg powder.

† Based on cell count (see p. 84).

‡ Based on 2-aminoethylphosphonic acid determination (see p. 85).

§ Helms & Rølle (1970).

Table 2. *The comparison of the relative nutritive value* of proteins in different food mixtures for fattening pigs, as determined microbiologically using* Tetrahymena pyriformis *W* (RNV_{pc} *and* RNV_{AEP}) *and their biological value* (BV) *and net protein utilization* (NPU) *assayed on rats*

(Mean values for two determinations)

Sample	*T. pyriformis* assay		Rat assay	
	RNV_{pc}† (*a*)	RNV_{AEP}‡ (*b*)	BV (*c*)	NPU (*d*)
High-protein concentrates				
M1	78·8	73·8	62·5	54·4
M10	69·1	66·5	61·3	54·5
M52	73·6	77·1	63·0	54·4
M64	69·6	74·6	59·3	49·6
Concentrates with decreased level of protein of animal origin				
M2	55·5	60·2	55·4	47·4
M65	72·9	73·2	60·2	49·8
M66	68·9	71·6	63·8	55·9
Plant mixtures				
M3	49·9	50·6	47·4	42·4
M4	46·5	41·9	52·7	43·1
M6	53·9	45·0	52·0	44·8
M11	46·0	44·3	53·3	46·9
Plant mixtures supplemented with lysine (Lys)				
M4+Lys	44·7	44·2	61·2	48·6
M6+Lys	69·7	68·3	60·4	52·6
Plant mixtures supplemented with methionine (Met)				
M6+Met	75·4	72·0	60·7	51·8
M11+Met	59·0	60·3	66·0	57·6
M67	69·6	72·8	63·3	52·5
Plant mixtures supplemented with both Lys and Met				
M6+Lys+Met	68·9	76·1	64·7	55·3
M11+Lys+Met	55·9	62·1	59·2	51·4
Mixtures containing lucerne (*Medicago sativa*)-protein concentrate Vepex§				
M53	74·7	68·6	58·3	50·1
M54	67·0	66·3	56·0	47·2

Correlation coefficients: *a,b*, 0·93; *a,c*, 0·59; *a,d*, 0·63; *b,c*, 0·67; *b,d*, 0·70; all at $P = 0·01$.

* Protozoa population with test protein:protozoa population with whole-egg powder.

† Based on cell count (see p. 84).

‡ Based on 2-aminoethylphosphonic acid determination (see p. 85).

§ Vepex, Budapest, Hungary.

Table 2 gives the comparison of RNV_{pc}, RNV_{AEP}, BV and NPU values for proteins in food mixtures. The highest values of RNV and BV were obtained for high-protein concentrates and plant mixtures supplemented with synthetic amino acids. An agreement between the results for food mixtures was not satisfactory. The results of RNV_{AEP} were very similar to NPU for plant mixtures only. For almost all other mixtures (Table 2) they were rather different (up to 20 units). On average protozoa reacted more effectively than rats to

Table 3. *Relative nutritive values as determined microbiologically using* Tetrahymena pyriformis *W (RNV$_{pc}$ and RNV$_{AEP}$) calculated for six foodstuffs of different protein quality*

(Mean values with their standard errors and standard deviations; values in parentheses are the number of replicates)

	RNV$_{pc}$*			RNV$_{AEP}$†		
Sample	Mean	SD	SE	Mean	SD	SE
Fish meal FM45	76·5 (7)	2·52	0·95	72·1 (6)	3·06	1·37
Blood meal BM61	25·8 (6)	0·45	0·18	27·0 (6)	0·62	0·25
Barley B41	43·7 (7)	2·43	0·92	46·3 (7)	2·52	0·95
Field bean (*Vicia faba*) FB51	45·3 (7)	1·65	0·62	47·0 (7)	0·85	0·32
Groundnut meal GN32	58·3 (5)	0·69	0·31	57·6 (5)	0·81	0·36
Soya-bean meal SB57	72·8 (7)	1·64	0·62	70·9 (2)	—	—

* Based on cell count (see p. 84).
† Based on 2-aminoethylphosphonic acid determination (see p. 85).

Table 4. *Mean dimensions (length × width; μm) and 2-aminoethylphosphonic acid-phosphorus content (AEP-P; μg/10^6 cells) of* Tetrahymena pyriformis *W cells as affected by different protein sources*

Sample	Cell dimensions (μm)	AEP-P
Whole-egg powder	56 × 29	4·24
Fish meal	53 × 25	4·28
Blood meal	47 × 28	3·94
Barley	48 × 24	4·06
Dried forage	42 × 24	3·37
Crude soya-bean meal	44 × 23	—
Crude soya-bean meal + Met	50 × 27	—
Toasted soya-bean meal	47 × 22	3·91
Toasted soya-bean meal + Met	54 × 27	4·27

Met, methionine.

amino acid supplements and protein of animal origin in food mixtures. The lack of response of *T. pyriformis* was observed only in one case, namely the M4 mixture + Lys, while rats reacted markedly (BV increased by about 20%). For mixtures supplemented with both Lys and Met RNV$_{AEP}$ were higher than RNV$_{pc}$ by approximately 11%.

A highly significant correlation (r 0·93, P = 0·01) was observed between RNV$_{pc}$ and RNV$_{AEP}$. The correlations between RNV$_{AEP}$ and both BV and NPU were rather poor, but correlation coefficients were slightly higher than between RNV$_{pc}$ and both BV and NPU (r 0·67, 0·70, 0·59, 0·63 respectively, all at P = 0·01).

Tests for the reproducibility of the different Tetrahymena methods showed (Table 3) that the standard error values of RNV$_{pc}$ for foods of different protein quality ranged from 0·18 for BM61 to 0·92 for B41. For RNV$_{AEP}$ standard errors ranged from 0·25 for BM61 to 0·95 for B41. The values of the coefficients of variation ranged between 1·2 and 5·6%.

Mean dimensions and AEP-P content of *T. pyriformis* cells grown on various foods are given in Table 4. Observation made over a period of several years on *T. pyriformis* growth in media containing various proteins confirmed the existence of differences in protozoal cell dimensions after 96 h incubation at 25° (Maciejewicz-Ryś, unpublished results). The largest were organisms grown on a whole-egg powder and the smallest were those grown on dried green forage (length and width shorter by 25 and 17·2% respectively). The supplementation of crude and toasted soya-bean with synthetic methionine resulted in the increase of the cell dimensions by 13·6–17·0 and 14·9–22·7% in length and width respectively. There was a parallel reduction in AEP-P content in the protozoal cells and cell size.

DISCUSSION

The study was aimed at finding whether the AEP content may be used as protozoal growth indicator in *T. pyriformis* assay of nutritive value of protein. Highly significant correlations observed between the RNV of proteins obtained by protozoa count and by AEP determination for both foodstuffs and food mixtures imply the possibility of using AEP as a new indicator of *T. pyriformis* W population growth in the protein evaluation assay.

Our observation that cell sizes of *T. pyriformis* grown in various media were quite different (Table 4) implied that the basal assumption of the counting method, i.e. equal amounts of synthesized protein in every protozoal cell (Fernell & Rosen, 1956) might be not always valid. The changes in *T. pyriformis* cell size according to the protein quality were also shown by Evancho, Hurt, Devlin, Landers & Ashton (1977). Summers (1963) found too, that if some essential amino acids were deficient in an incubation medium the volume of *T. pyriformis* cells was decreased by one-third. McCashland & Johnson (1957) emphasized the fact that in most published reports only protozoa count was taken into consideration while the process of growth consists in the increase of the total cell number as well as the size and the protoplasm content of micro-organisms. The latter two factors are strongly related to the feeding level (McCashland & Johnson, 1957) and the source of energy (Reynolds, 1970).

Because a decrease of the size of *T. pyriformis* cells was accompanied by their lower AEP-P content (Table 4), we expected that the RNV calculated on the basis of AEP would be independent from differences in cell sizes and correlated more closely with the results of tests with rats. Though such a trend could be observed, still the correlation between the results of the microbial method and the tests with rats was comparatively poor. The reason for this could lie in discrepancies between the results of RNV and BV for some foodstuffs and food mixtures. Thus large differences were found for meat and bone meals. Similarly, Boyne, Carpenter & Woodham (1961), comparing RNV results with NPU for meat as well as for meat-and-bone meals observed poor correlation between these values. It might have been caused by inability of the micro-organisms to distinguish between Lys and δ-hydroxy-Lys (Carpenter, 1973), both of which are present in collagen. Omitting the meat-and-bone meals from the calculations (Table 1) resulted in an increase of the correlation coefficient between BV and RNV for foodstuffs, from 0·67 to 0·90. Discrepancies observed between the results of evaluation of food mixtures using rats or *T. pyriformis* might have also been caused by quantitative differences in the requirements for different amino acids.

Rølle & Eggum (1971), studying the relationship between RNV and BV for sixty foodstuffs and food mixtures, found low correlation (r 0·22) for foods with low sulphur amino acid content. For foods rich in these compounds (level higher than 3·4 g/16 g N), the correlation between RNV and BV was significant (r 0·70).

The results obtained show that *T. pyriformis* reacts differently from rats to changes in food quality resulting from differences in amino acid composition (Rølle, 1975) or trypsin inhibitor

activity (Viswanatha & Liener, 1955). Therefore the results of the microbial method and tests with rats may be different.

The *T. pyriformis* assay involves less time and effort than biological tests with rats, and it has found application in the estimation of nutritive value during recent years (Frank, Baker, Hutner, Rusoff & Morck, 1975; Landers, 1975; Evancho *et al.* 1977). However, the method in its present state of development has defects that make it unreliable for food mixtures and also limit its usefulness in the evaluation of foodstuffs.

Replacing direct counting of protozoa cells by indirect AEP determination did not result in any marked improvement of the correlation of RNV with BV or NPU, but the modification avoids tedious cell counting in a haemocytometer and allows analyses to be done at any time. It may be usefully applied in the *T. pyriformis* assay of amino acid availability (Stott & Smith, 1966; Shorrock, 1976; El-Sherbiny, Draper & Topps, 1976).

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